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CORRIGENDA

BOYNE, A. W., EADIE, J. M. & RAITT, K. (1957).
J. gen. Microbiol. 17, 414–423

On page 420, the legend to Fig. 2 *should read:*

Concentration of 'large' and 'small' organisms in rumen contents of a sheep sampled on different days. 'Large' organisms sampled at 10 a.m. = ×, at 3 p.m. = +; 'small' organisms sampled at 10 a.m. = ○, at 3 p.m. = ●.

THORNE, C. B. & BELTON, F. C. (1957). *J. gen. Microbiol.* 17, 505–516

On page 512, line 1:

For: 'that $FeSO_4$ need not be omitted from the medium,'

Read: 'that $FeSO_4$ could be omitted from the medium,'

PEREIRA, H. G. & KELLY, B. (1957). *J. gen. Microbiol.* 17, 517–524

On page 517, last line of Summary:

For: 'the latter to inactivation by ultraviolet light'

Read: 'the former to inactivation by ultraviolet light'

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MacLennan, J. D., Mandl, I. & Howes, E. L. (1958). *J. gen. Microbiol.* 18, 1–8

New Proteolytic Enzymes from *Clostridium histolyticum* Filtrates

By J. D. MacLENNAN, INES MANDL and E. L. HOWES

Departments of Surgery and Microbiology, College of Physicians and Surgeons, Columbia University, New York

SUMMARY: Oakley & Warrack (1950) established the presence of two serologically distinct proteolytic enzymes in *Clostridium histolyticum* filtrates, a β-toxin digesting native collagen and a cysteine-activated γ-toxin attacking azocoll and other protein substrates.

In the present paper we give evidence of another proteolytic enzyme, not activated by cysteine and serologically different from those previously described. This we designate as δ-toxin. We also indicate the possibility of the presence of a fourth enzyme more stable than the others and active against various synthetic substrates in conjunction with cysteine. This enzyme could not be inhibited by antisera. Furthermore peptidases have been detected and will be described in detail elsewhere.

Some of the differences between these enzyme systems are discussed.

Several papers have been published on the various toxins and enzymes present in culture filtrates of *Clostridium histolyticum*, the most important studies being those of Oakley & Warrack (1950) who were able by serological methods to define three factors: the α-toxin, which is the main lethal toxin, the β-toxin, a collagenase, and the γ-toxin, which is a cysteine-activated proteinase. Bowen (1952) and Howard (1953) published evidence for a distinct haemolysin component antigenically related to *C. welchii* θ-toxin and to tetanolysin. There has also been considerable interest in the biochemical nature of the proteolytic enzymes. Since the demonstration by Jennison (1947) that culture filtrates of *C. histolyticum* were collagenolytic some degree of concentration of the collagenase has been obtained by various methods (Tytell & Hewson, 1950; Mandl, MacLennan & Howes, 1953; DeBellis, Mandl, MacLennan & Howes, 1954) but the exact nature of this enzyme remains uncertain. On the other hand, very considerable purification of the cysteine-activated proteinase (the γ-toxin) has been achieved (Kocholaty & Krejci, 1948; Lepow, Katz, Pensky & Pillemer, 1952; Ogle & Tytell, 1953). All these studies confirm that this enzyme is cysteine-activated and readily inhibited by iodoacetamide and similar compounds. It was therefore with considerable surprise that in the course of our investigations of clostridial proteinases (MacLennan, Mandl & Howes, 1953; Mandl, MacLennan & Howes, 1953) we separated from *C. histolyticum* filtrates a proteinase which was not activated by –SH compounds. In a previous publication (Mandl *et al.* 1953), we briefly mentioned that this new enzyme was distinct from either the β- or γ-toxins of Oakley (Oakley & Warrack, 1950) and suggested for it the name 'δ-toxin'. The present paper is concerned with a more detailed account of these and subsequent studies.

RESULTS

Professor C. L. Oakley very kindly put at our disposal six sera (2001, 2005, 2008, 2035, 8003, 8006) which had been calibrated by him (Oakley & Warrack, 1950). These sera in various dilutions were incubated for 30 min. at 37° with solutions of different enzyme preparations. The mixtures were then added to 10 mg. amounts of azocoll (Oakley, Warrack & van Heyningen, 1946) and the volumes made up to 10 ml. with phosphate saline buffer (pH 7·4) by an adaptation of the procedure of Oakley *et al.* (1946). The colour released was read in a Klett colorimeter after 24 hr. of incubation at 37° and the units required for complete inhibition calculated by comparison with one serum (2001), to which a value of 100 enzyme units had arbitrarily been assigned. Table 1 shows the values so obtained against two different proteinase preparations (Mandl *et al.* 1953) as compared with the anti-β and anti-γ values obtained by Oakley & Warrack (1950) with these same sera. Almost the same relative figures were obtained when azocoll tests were run with 50 mg. amounts of substrate and correspondingly higher enzyme concentrations on a Fisher Gyrosolver shaking machine for 15 min. Professor Oakley has since very kindly checked our results and essentially confirmed them. A more detailed report of his investigations is given in the following paper (Oakley & Warrack, 1958).

Table 1. *Values of antisera against proteinase preparations* (*anti-δ units*) *compared with Oakley's anti-β and anti-γ units*

Serum no.	Anti-δ units against proteinase no. 199	Anti-δ units against proteinase no. 245	Anti-β units Oakley & Warrack (1950)	Anti-γ units Oakely & Warrack (1950)
RR 2001	100	100	100	100
RR 2005	75	50	350	160
RR 2008	170	150	370	360
RR 2035	200	175	230	150
RR 8003	120	125	260	550
RR 8006	120	100	180	410

Table 1 clearly shows that the main component of our proteinase preparations [P 199, P 245] (Mandl *et al.* 1953) is not identical with either the β- or γ-toxin of Oakley & Warrack (1950). We proposed the designation of δ-toxin for this enzyme (Mandl. *et al.* 1953).

Similar tests were set up to determine the immunological identity of the collagenase in our collagenase preparations [K 240, K 265, K 210] (MacLennan *et al.* 1953; Mandl *et al.* 1953). For these studies, in addition to azocoll, purified collagen (Einbinder & Schubert, 1951) was used as a specific substrate, complete inhibition of digestion being taken as the end-point. As will be seen from Table 2, values not too different from Oakley's anti-β figures (Oakley & Warrack, 1950) were obtained.

With azocoll as a substrate on the other hand (last column of Table 2), only four of the six sera show end-points within the range of experimental error for β values. Sera nos. 2005 and 2035 approached γ end-points far more closely. Sera nos. 2001 and 2008 may of course be β or γ, though sera nos. 8003 and 8006 could never be γ (Table 1). It is not surprising that the antibody present in lesser amounts (β in no. 8003 or no. 8006, γ in no. 2005 or no. 2035) should determine the end-point, if a mixture of these enzymes is assumed. There are no δ end-points with these preparations and the relative values are the same for a crude collagenase with 35–40 % proteinase (δ) and purified material completely free of activity against casein, though not against benzoyl arginine amide. (See below and DeBellis *et al.* 1954).

Table 2. *Values of antisera against collagenase preparations compared to anti-β units*

Serum no.	Collagen substrate against collagenase no. 240	Anti-β Oakley & Warrack (1950)	Azocoll substrate against collagenase no. 265
RR2001	100	100	100
RR2005	400	350	125
RR2008	350	370	400
RR2035	300	230	75
RR8003	250	260	200
RR8006	220	180	150

Chemical tests confirmed that more than one enzyme distinct from collagenase was present in our proteinase preparations. Three typical experiments may be described.

(*a*) When the enzyme preparation was heated to 56° in a water-bath for 30 min., activity against azocoll was decreased to about one-third of its original value. Neither of these activities was affected by adding cysteine or iodoacetate in final concentrations of less than 0·01 M, while larger amounts of cysteine inhibited both the heated and unheated sample. Activity against collagen was lost almost completely by this heat treatment. On the other hand, activity against the synthetic substrate benzoyl arginine amide (B.A.A.) and arginine methyl ester (A.E.) was retained without any loss. These substrates were attacked only in the presence of cysteine, and optimum conditions were the same before and after heating. Table 3 gives the values obtained in two typical experiments

Table 3. *Loss in enzymic activity after heating proteinase and collagenase preparations* 30 *min. at* 56°

	Relative activites against different substrates			
Enzyme preparations	Azocoll	Collagen	Benzoyl arginine amide	Arginine ester
P199	100	0	100	100
P199 heated	38	0	100	100
K210	100	100	—	—
K210 heated	12	10	—	—

Our methods for carrying out these tests were described in greater detail elsewhere (Mandl *et al.* 1953).

The amount of collagen digested was measured by a colorimetric ninhydrin reaction with the supernatant fluid (Harding & MacLean, 1915; Harding & Warneford, 1916). Benzoyl arginine amide was split by the enzyme into benzoyl arginine and ammonia, the ammonia being then trapped in boric acid by Conway diffusion (Conway & Byrne, 1933) and estimated with Nessler's reagent (Mandl & MacLaren, 1949). In the case of arginine methyl ester the amount of unchanged ester substrate was determined by Hestrin's alkaline hydroxylamine method (Hestrin, 1949).

The difference in stability of the various factors indicates the presence of at least two enzymes. It seemed possible that this more stable enzyme, active against the synthetic substrates in the presence of cysteine, might be the γ-toxin (Oakley & Warrack, 1950). This hypothesis was tested by repeating the serological experiments outlined on page 2 of this paper, using heated enzyme against the six sera put at our disposal by Professor Oakley, with and without added cysteine. In typical experiments half as much serum was required for the neutralization of the azocoll activity of P199 after heating than before heating but comparison of the end-points obtained gave exactly the same relative values as those shown for δ-enzyme in Table 1. Unlike Oakley & Warrack, (1958), we did not find that the presence or absence of cysteine made any difference in the relative units required for complete inhibition.

(*b*) Differential inactivation very similar to that obtained on heating was also observed after lowering the pH value of enzyme solutions to pH 4·5 (in KH_2PO_4), leaving it at that pH value for 1 hr. or overnight, then adding Na_2HPO_4 to bring the pH value back to 7·4 and retesting. Again azocoll activity decreased to one-third or less, while the activity against the synthetic substrates was retained in full. This experiment was also carried out with a crude *Clostridium histolyticum* Seitz filtrate, the pH value being decreased by adding HCl. This preparation lost all its collagenase activity, and almost all its azocoll activity, while benzoyl arginine amide was hydrolysed as before. The result appears of interest also in view of the fact that Lepow *et al.* (1952) made use of low pH values in the precipitation of their proteinase (presumably γ). This seems further proof that the main constituent of our enzyme cannot be γ-toxin but must be some other more labile enzyme (δ). Whether the remaining activity against benzoyl arginine amide is due to γ seems doubtful (DeBellis *et al.* 1954). However, the small amount of azocoll activity remaining was not increased by adding cysteine, and this fact as well as the end-points obtained in serological tests were not in favour of such an assumption. Hydrolysis of protein substrates other than azocoll, e.g. casein, was retarded in the same way as with azocoll and were also unaffected by cysteine. Table 4 shows some comparative results. Recently we have obtained evidence that refractionation of crude collagenase preparations gave fractions of maximum activity against benzoyl arginine amide, that did not attack casein (or haemoglobin). Such fractions sometimes attacked casein in the presence of added cysteine, probably due to the presence of small amounts of γ-enzyme. Similarly, purified β-enzyme (collagenase) had no effect on these two substrates (DeBellis *et al.* 1954).

Table 4. *Loss in enzymic activity of a proteinase preparation after heating or acidification, tested with and without added cysteine*

Enzyme preparation P199	Relative activities against substrates			
	Casein	Azocoll	Benzoyl arginine amide	Arginine ester
Enzyme 199	100	100	0	0
+Cysteine	100	100	100	100
Heated 30 min. 56°	62	34	0	0
Heated+cysteine	53	34	100	100
pH 4·5 24 hr.	48	16	0	0
pH 4·5+cysteine	47	16	100	90

Casein hydrolysis was tested by a modification of Grob's method (1946) of determining the amount of undigested casein by the turbidity resulting when a sample was added to a mixture of 20 % sulphosalicylic acid: 25 % HCl (2:1). Essentially the same serological tests as those already described for the heated enzyme were performed with proteinase preparation which had been partly inactivated by decreasing the pH value. The results again seemed to indicate the δ end-points.

(*c*) When a 1 % solution of the same proteinase preparation percolated through an ion exchange resin (Rohm and Haas XE 64), the resulting percolate did not show any activity against azocoll but against benzoyl arginine amide and arginine ester activity was as high as that of the original solution. Addition of cysteine, Fe^{++}, Fe^{++}+cysteine or Ca^{++} to the percolate had no effect on its ability to digest azocoll nor could this activity be recovered by mixing the percolate and an eluate obtained by passing 10 % NaCl through the column. Analysis for protein showed no substantial loss following passage through the column, so that partial inactivation rather than separation seemed to have taken place.

Collagenase solutions subjected to the same treatment retained some activity (one-third to two-thirds) against azocoll, but lost nearly all their collagenolytic powers.

To summarize conclusions drawn from these three experiments, it seems obvious that the main constituent of our preparations is less stable than that described by other workers (Lepow *el al.* 1952; Ogle, 1952). Ogle (1952) reported his cysteine-activated proteinase to be stable at 50° for 30 min. and as mentioned above, Lepow *et al.* (1952) precipitated their enzyme at pH 4·5. γ-Toxin differs from ours (δ) both serologically and in its susceptibility to activators and inhibitors. While hydrolysis of azocoll or casein by our proteinase is inhibited or unaffected by cysteine or Fe^{++}, Ca^{++} has a strong activating effect (Mandl *et al.* 1953).

So far as we know, the identity of the cysteine activated enzyme (Kocholaty & Krejci, 1948; Lepow *et al.* 1952; Ogle & Tytell, 1953) with each other and Oakley's γ-toxin has not been tested by serological means.

Our enzyme preparations show cysteine-activation only towards synthetic substrates and the factor responsible for this activity is relatively stable and distinct from the δ-enzyme. In fractionation experiments it accompanies the collagenase (β) rather than δ-proteinase (DeBellis *et al.* 1954). Loss in activity cannot be accounted for by collagenase instability alone since collagenase-free proteinase behaves in the same way. In at least some cases all azocoll activity is lost without affecting the activity against benzoyl arginine amide and arginine ester; since γ-enzyme attacks azocoll, the stable factor is probably not γ. To our knowledge, all known proteinases are active against azocoll.

Many attempts were made to show the presence of trace amounts of γ-toxin. The neutralization points of the six sera against proteinase plus cysteine and against proteinase plus iodoacetic acid were investigated in the hope of getting some indications on this point. We have to admit, however, that some of these titrations were very erratic and had to be discarded, and even at best end-points were not as consistent as those obtained with the enzyme alone. So far as conclusions can be drawn, there was no marked difference between the relative amounts required in each series, and neutralization points were approximately the same and essentially δ end-points. We understand that Professor Oakley in his more extensive investigations did observe differences pointing to the presence of traces of γ and that he will report on his findings in the following paper (Oakley & Warrack, 1958). We can detect a cysteine-activated casein-splitting enzyme, possibly γ, in fractions free of δ-proteinase, usually precipitated with collagenase in ammonium sulphate fractionations (DeBellis *el al.* 1954).

The question of identity of the benzoyl arginine amide hydrolysing enzyme with Oakley's γ-toxin would have been amenable to serological solution if the end-points for the inhibition of its action against synthetic substrates by various sera could have been compared. Experiments were set up to this end, but it was soon found that even very large excess of antiserum had no effect on these reactions. Other cases are known in which enzymes (e.g. urease) cannot be inhibited by their antisera, even where definite enzyme-antienzyme complexes are formed; for a review see Cinader (1953). Bidwell & van Heyningen (1948) observed a similar case with *C. welchii* κ-toxin; the action of this enzyme against gelatin was not affected by sera inhibiting collagen and azocoll activity. Our gelatinase, however, can be inhibited by sera (Mandl & Zaffuto, 1958). Bidwell & van Heyningen, after considering possible reasons, found the most likely explanation in the fact that the comparatively small molecules of gelatin were not prevented from reaching the catalytic centre of the enzyme, whereas the gross particles of collagen or azocoll were held back. Such a conclusion could account for the failure of our sera to inhibit the hydrolysis of the much smaller benzoyl arginine amide and arginine ester molecules. It also agrees with the statements of Cinader (1953) and Marrack (1950) that the probability of inhibition is greater the larger the substrate. The small size of the synthetic substrate molecule would then be the cause of the lack of inhibition. This explanation obviously does not answer the question whether a new enzyme is involved.

However, if all the findings are considered together, it seems likely that a distinct fourth enzyme (ϵ?) is responsible for the amidase-esterase activity, irrespective of whether γ is present in traces or not. One or more further components have recently been detected, both chemically (DeBellis *et al.* 1954) and serologically. They attack a variety of synthetic peptide substrates and are described in detail elsewhere (Mandl *et al.* 1957).

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The Cysteine-Inactivated Proteinase (δ-antigen) of *Clostridium histolyticum*

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SUMMARY: Evidence is provided in support of the view of MacLennan, Mandl & Howes (1958) that some strains of *Clostridium histolyticum* produce a cysteine-inactivated proteinase—the δ-antigen.

Three soluble antigens in *Clostridium histolyticum* filtrates have been differentiated by immunological means: the necrotizing and lethal toxin α, the collagenase β, and the cysteine-activated proteinase γ. Though the existence of a cysteine-inactivated proteinase had been demonstrated by van Heyningen (1940), Oakley & Warrack (1950) were unable to show its presence in the culture filtrates they had used to differentiate the α-, β- and γ-antigens. Since then Mandl, MacLennan & Howes (1953) and MacLennan, Mandl & Howes (1958) have found a cysteine-inactivated proteinase (δ-antigen) in some filtrates of *C. histolyticum* cultures examined by them, and we here provide some evidence to support their views.

METHODS

Culture filtrate preparations. Through the courtesy of Dr J. D. MacLennan we received six dried partly purified preparations from filtrates of *Clostridium histolyticum*: P199, P208, P235, P245, P278 and P279.

Antisera. We used the six *Clostridium histolyticum* antisera referred to by Oakley & Warrack (1950).

Diluents. A mixture of equal volumes of sodium chloride solution (1 %, w/v) and broth (broth + saline) was used as a non-activating diluent. For activation with cysteine the culture filtrate preparations were dissolved in 0·1 M-cysteine (neutralized) made by dissolving cysteine in a little distilled water, adjusting the pH value to 7·1–7·3 (checked electrometrically) and diluting the mixture to 0·1 M-cysteine with distilled water.

Assay of antisera. The antisera were assayed by conventional methods against RR 2001 (100 units/ml.) as standard, with azocoll as indicator (Oakley, Warrack & van Heyningen, 1946). The level of test was 0·25 unit, and the standard indicating effect was the release of a certain amount of 'colour' from the azocoll. A suitable end-point tube was chosen from the assay of the standard serum that was always included in any set of tests, and used to determine the end-point, by interpolation when necessary, in tests of other sera. In tests with cysteine-activated filtrates, the tubes should be filled nearly to the top to prevent oxidation of the cysteine. The values given in the Tables are the average of at least three determinations; the error of a single test appears to be about 20 %.

RESULTS

Activity of the preparations

All the preparations, dissolved in broth + saline, rapidly dissolved azocoll, releasing 'colour'; they had little or no effect on collagen paper, and no necrotizing effect on intradermal injection into guinea-pig's skin. Solution in cysteine increased the effect on azocoll of all preparations except P245, whose activity was decreased.

Effect of cysteine on the test dose of the filtrate preparations

As Table 1 shows, the test doses of these preparations were invariably altered by solution in cysteine, as compared with those of material dissolved in broth + saline. In all but P245 the test dose was much decreased; in P245 it was increased twofold. Oakley & Warrack (1950) have already shown that the gamma antigen of *Clostridium histolyticum* is activated by cysteine, so that solution in cysteine, if it affected the test dose at all, would be expected to decrease it, if gamma were the only antigen affected. The fact that the test dose of P245 was increased by solution in cysteine suggests that a cysteine-inactivated antigen was present in the preparation.

Table 1. *Test doses of* Clostridium histolyticum *filtrate preparations dissolved in broth + saline or in neutralized* 0·1 M-*cysteine*

Filtrate preparation	Test dose (mg.) of preparation dissolved in		Ratio of $\frac{\text{test dose in broth saline}}{\text{test dose in cysteine}}$
	Broth + saline	Neutralized 0·1 M-cysteine	
P199	0·125	0·062	2
P208	0·125	0·018	7
P235	0·44	0·021	21
P245	0·38	0·68	0·5
P278	0·20	0·023	9
P279	0·20	0·022	9

Table 2. *Values of antisera against* Clostridium histolyticum *filtrate preparations dissolved in broth + saline, with azocoll as indicator*

Serum	Values of sera against								
	α-antigen	β-antigen	γ-antigen	P199	P208	P235	P245	P278	P279
RR2001*	100	100	100	100	100	100	100	100	100
RR2005	240	350	160	90	90	80	75	70	80
RR2008	27	380	360	165	170	40–160†	175	150	160
RR2035	570	250	150	200	185	100–400	185	170	200
RR8003	55	210	550	125	125	60–240	120	130	120
RR8006	73	160	410	150	140	80–320	150	140	140

* Standard. † Inadequate amounts of preparation for closer test.

Values of antisera against partly-purified filtrate preparations dissolved in broth + saline

Table 2 gives the values of the six antisera against the known *Clostridium histolyticum* antigens for comparison with their values against the purified preparations. It is clear that the latter figures bear no relationship to the values against the α-, β- or γ-antigen; we may therefore postulate another antigen (δ) as that responsible for the end-points in these tests.

Effect on antiserum values of solution of the filtrate preparations in cysteine

As Table 3 shows, solution of the preparations in cysteine had a considerable effect on the antisera assayed against them. Most of the values obtained in these tests agree very well with the anti-γ values of the antisera, if one allows for the accuracy of the tests; the values for antiserum RR 2005, and that for antiserum RR 8003 against filtrate preparation P 245, are however, rather lower than the known anti-γ values, and may be due to some other antigenic proteinase. When these results are compared with those for the change in test dose produced by solution in cysteine, it is clear that delta antigen is inactivated by cysteine.

Table 3. *Values of antisera against* Clostridium histolyticum *filtrate preparations dissolved in neutralized* 0·1 M-*cysteine, with azocoll as indicator*

	Values of sera against								
Serum	α-antigen	β-antigen	γ-antigen	P 199	P 208	P 235	P 245	P 278	P 279
RR 2001*	100	100	100	100	100	100	100	100	100
RR 2005	240	350	160	110	115	115	100	125	120
RR 2008	27	380	360	415	475	475	310	530	510
RR 2035	570	250	150	230	220	230	180	275	245
RR 8003	55	210	550	530	630	710	380	750	650
RR 8006	73	160	410	350	390	470	300	450	400

* Standard.

DISCUSSION

It is evident that when a culture-filtrate preparation contains a cysteine-activated and a cysteine-inactivated proteinase, solution of the preparation in cysteine will increase its activity, leave it unaffected, or decrease it, depending on the relative amounts of the two components and on the concentration of activating substances present. If we take the test doses of the filtrates in broth + saline as inversely proportional to their δ-antigen content, and those in cysteine as inversely proportional to the γ-antigen content, it is clear that a very considerable degree of purification has been obtained in some of them. Thus, at one end of the scale filtrate preparation P 245 has a γ:δ ratio of about 0·5:1, whereas filtrate preparation P 235, at the other, has a γ:δ ratio of 21:1, a forty-fold increase in purity for γ-antigen. The other preparations show intermediate increases in purity. Obviously the presence of other reducing agents in the broth + saline solutions may decrease their δ activity slightly, and thus slightly alter these ratios, but judging from the amount of cysteine necessary for complete inactivation of δ-antigens, this effect is very small. The δ-antigen is not destroyed by cysteine, for when the cysteine is oxidized by shaking the mixture with air the δ values are obtained when the shaken preparation is being used as antigen in serum assays.

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Serological Evidence for a Specific *Clostridium histolyticum* Gelatinase

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SUMMARY: Evidence is presented that a specific gelatinolytic enzyme besides collagenase is present in purified *Clostridium histolyticum* collagenase preparations. This means that collagenase itself need not be able to digest gelatin but it does not exclude the possibility.

The purest collagenase available to date (DeBellis, Mandl, MacLennan & Howes, 1954) is free of non-specific proteolytic activity and active only against collagen and collagen degradation products (azocoll, gelatin). The question whether these activities are due to the same enzyme, i.e. whether collagenase will break down gelatin as well as collagen is still unsolved. It is possible that a series of reactions catalysed by distinct enzymes is involved but that our analytical procedures are inadequate for the detection of the primary effect of collagenase. This may involve simple breaking of hydrogen bonds as in urea treatment which results in denatured collagen susceptible to enzymes such as trypsin. However, the entire collagenase system differs from such proteinases by its specificity and complete lack of reaction with other proteins such as casein or haemoglobin as well as numerous synthetic substrates tested in this laboratory (Mandl, Zipper & Ferguson, 1957).

The presence of a specific gelatinase was first suspected when fractionation of crude collagenase (DeBellis *el al.* 1954) invariably showed the fraction with maximum collagenase activity preceding the corresponding azocoll maximum. If the collagenase were the only enzyme in this region capable of attacking azocoll, the maxima should coincide. Furthermore, the ratio of collagenase to azocoll activity is not the same in different preparations and the concentrations of certain ions (Co, Ni) needed to inhibit activity of any one preparation differ with the substrate hydrolysed (see Table 1).

Ogle & Logan (1956) have also advanced evidence for the presence of a distinct gelatinase in the collagenase system.

Gelatin or azocoll attack by the preparations investigated could not be ascribed to other known proteinases (MacLennan, Mandl & Howes, 1958; DeBellis *et al.* 1954) since the fractions were free of δ-proteinase and γ- (or ϵ–) proteinase would not be active in the absence of cysteine.

In the belief that serological tests would be more conclusive the following set of experiments was set up: varying dilutions of six sera kindly supplied by Professor Oakley of Leeds University and used for the detection of other specific enzymes (MacLennan *et al.* 1958; Oakley & Warrack, 1950) were incubated for half-hour periods with equal volumes of 0·1 % solutions of an enzyme fraction rich in collagenase and free of δ-proteinase prepared according

Table 1. *Molar concentrations of different metal ions completely inhibiting action of* 0·1 *mg enzyme L* 139$_p$28 *fraction III* [26–30 % $(NH_4)_2SO_4$ *conc.*]

	Substrates		
Metal ion	Collagen (10 mg/2·5 ml.)	Azocoll (50 mg./10 ml.)	Gelatin (30 mg./2 ml.)
Ca	$>0·5$	10^{-1}	>1
Co	0·5	10^{-3}	$>0·5$
Fe^{++}	10^{-3}	10^{-3}	0·5
Fe^{+++}	10^{-3}	10^{-3}	10^{-2}
Mg	0·5	10^{-1}	>1
Mn	0·5	10^{-2}	$>0·5$
Ni	10^{-3}	10^{-3}	$>0·5$
Pb	10^{-3}	10^{-3}	10^{-1}
Zn	10^{-3}	10^{-3}	10^{-1}

Table 2. *Relative serum concentrations completely inhibiting action of enzyme No. L* 139$_p$28 *fraction III* [26–30 % $(NH_4)_2SO_4$ *conc.*]

	Substrate		
Serum no.	Collagen (10 mg./5 ml.)	Azocoll (10 mg./5 ml.)	Gelatin (30 mg./ml.)
RR 2001	100	100	100
RR 2005	300	200	130
RR 2008	300	400	155
RR 2035	200	200	155
RR 8003	100	300	210
RR 8006	150	200	130

to the method of DeBellis *et al.* (1954), The resulting complexes were then allowed to react with the substrates under investigation—collagen, azocoll and gelatin—and the lowest serum dilutions which completely inhibited the breakdown of these substrates compared. Azocoll and collagen tests were performed as previously described (Mandl, MacLennan & Howes, 1953; MacLennan *et al.* 1958). Gelatinase was tested by its action on 3 % gelatin in 0·1 M-phosphate buffer pH 7·1. In all tests with added metal ions (Table 1) veronal + acetate buffer pH 7·4 (Michaelis, 1931) was used instead of phosphate. After incubation at 37° for 3 hr. the tubes were placed in the refrigerator and the end-point determined visually as the lowest serum concentration preventing liquefaction. Relative results are shown in Table 2, arbitrarily assigning a value of 100 to all anti-enzyme concentrations of serum no. 2001.

Relative inhibiting concentrations for azocoll and collagen hydrolysis largely agree with those found by MacLennan *el al.* (1958) for crude collagenase, but there are some differences, i.e. relative values of serum RR 8003 for collagen and RR 2035 for azocoll, between the crude collagenase and the purified fraction used in this study. Anti-collagenase values with collagen as substrate are essentially identical with the values of Oakley & Warrack (1950). With azocoll as substrate, values differ for some sera, indicating the presence of another enzyme, besides collagenase, capable of attacking this substrate.

The newer results with gelatin as substrate show that some other enzyme besides that acting on collagen is present and active against gelatin, although the possibility that the collagenase (β-toxin) also acts against gelatin cannot be excluded.

Table 3 shows a comparison between the end-points against azocoll and gelatin substrate of a purified δ-proteinase fraction, free from collagenase. Serum no. 2001 has again been arbitrarily assigned relative values of 100.

Table 3. *Relative serum concentrations completely inhibiting action of enzyme No.* $L\,139_p28$ *fraction I* [0–22 % $(NH_4)_2SO_4$ *conc.*]

	Substrate	
Serum no.	Azocoll (10 mg./5 ml.)	Gelatin (30 mg./ml.)
RR 2001	100	100
RR 2005	50	75
RR 2008	200	115
RR 2035	200	170
RR 8003	100	100
RR 8006	100	115

The end-points agree with the δ values found with the differently prepared and far less active proteinase described by MacLennan *et al.* (1958). They are the same against both substrates within experimental error and distinct from those shown in Table 2. The gelatinase activity of this fraction can therefore be ascribed to δ-proteinase and is distinct from the new gelatinase reported here.

The findings reported increase the minimum number of proteolytic enzymes present in *Clostridium histolyticum* filtrates to seven: δ-proteinase (cysteine inhibited), γ-proteinase (cysteine activated), β-collagenase, amidase-esterase (ϵ?), at least two peptidases (Mandl, Ferguson & Zaffuto, 1957) and the new gelatinase here reported. They also show that a specific gelatinase is associated with the purest collagenase available. Collagenase itself may or may not attack gelatin.

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Acytostelium leptosomum: A Unique Cellular Slime Mould with an Acellular Stalk

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SUMMARY: A new genus and species of the Acrasieae is described, and for this the binomial *Acytostelium leptosomum* is applied in recognition of its acellular sorophores and the diminutive proportions of its sorocarps. This slime mould has been isolated upon four occasions from soil and forest litter collected from deciduous forests within the United States. The vegetative stage of *A. leptosomum*, as that of the other cellular slime moulds of this Class, consists of the independent growth of myxamoebae which feed upon bacterial cells. The fruiting stage likewise duplicates that of other members of the Acrasieae in its early manifestations; but it differs markedly from these in its terminal phase, i.e. the construction of the mature fructification, or sorocarp. *A. leptosomum* is at present unique among these slime moulds in its capacity to produce a sorocarp with a non-cellular stalk. The morphogenetic process whereby this is accomplished is not thoroughly understood; nevertheless, a possible explanation of how the stalk may be formed without the expenditure of any cells is offered.

In his *Monograph of the Acrasieae*, representing the only comprehensive taxonomic treatment of these simple cellular slime moulds, Olive (1902) recognized three familes, namely: the Sappiniaceae, the Guttulinaceae, and the Dictyosteliaceae. The vegetative stage in each was noted to consist of independent free-living myxamoebae, whereas the fruiting stage was marked by the aggregation of these myxamoebae into multicellular organizations, termed pseudoplasmodia, and by their subsequent individual but co-ordinated differentiation to construct fructifications of varying complexity. Olive questioned whether the first of these families, represented only by the genus *Sappinia* Dangeard (1896), should be included since it showed no evidence of a well-defined pseudoplasmodium. The myxamoebae, in fruiting, simply collected on projections above the substratum, or as individuals, developed short pedicels which elevated the resting cells above the underlying substratum.

The second family, the Guttulinaceae, was thought to represent a limited advance. Following vegetative growth, the myxamoebae formed small if somewhat ill-defined psuedoplasmodia and in the ensuing fructifications exhibited some divergent cellular differentiation. Two genera were included. *Guttulinopsis* Olive (1901), regarded as the more primitive, formed small mound-like fructifications consisting simply of encysted individual myxamoebae, termed pseudospores. The cells of the stalk (or basal portion) were slightly elongate. The older genus, *Guttulina* Cienkowsky (1873), formed fructifications* consisting of spore masses, or sori, composed of rounded to angular spores with true walls, and stalks composed of wedge-shaped cells that were only slightly differentiated. The myxamoebae of both genera were characterized by lobose pseudopodia.

The third family, the Dictyosteliaceae, typified by Brefeld's genus *Dictyostelium* (1869) was, as the name implies, characterized by fructifications with prominent stalks which presented a net-like appearance, a condition resulting from the extreme vacuolation and compaction of their constituent cells. The vegetative myxamoebae possessed short, pointed (filose) pseudopodia. Four genera were included, and all were characterized by sorocarps exhibiting an abrupt separation into sterile supportive stalk tissue and strongly differentiated propagative cells, or spores. Two of these genera are to-day well known: *Dictyostelium*, distinguished by simple or irregularly branched sorophores bearing globose sori; and *Polysphondylium* (Brefeld, 1884), characterized by symmetrically branched (whorled) fructifications in which the main axis and each lateral branch bears a globose sorus. The genus *Acrasis*, known only from van Tieghem's description (1880), was reported to produce simple or clustered fruiting structures composed of a single tier of sterile, vacuolate stalk cells bearing a chain of echinulate spores. The remaining genus, *Coenonia*, likewise described by van Tieghem (1884) and not since rediscovered, was said to produce simple or branched fructifications. The main axis and each lateral branch (when present) bore a sorus in a small cupule composed of highly differentiated cells.

Thus in the families and genera which comprise the primitive class Acrasieae, Olive (1902) discerned an evolutionary sequence of increasingly advanced forms progressing from Sappinia through Guttulinopsis and Guttulina to Acrasis, Dictyostelium, Polysphondylium and Coenonia. Whereas markedly different levels of sorocarp complexity and cellular specialization are attained in the completed fructifications of these genera, the singular characteristic of the entire group is the co-ordinated behaviour and ultimate differentiation of individual amoeboid cells as the structural elements responsible for effecting whatever generic and specific developmental patterns are achieved.

A few years ago a diminutive slime mould was discovered which obviously belongs to the Acrasieae, yet differs from previously described genera by producing fructifications with non-cellular stalks, or sorophores. The vegetative stage consists of free-living myxamoebae and is indistinguishable from that of a Dictyostelium or a Polysphondylium; and the radiate pseudoplasmodia composed of streams of aggregating myxamoebae are essentially similar to those seen in the smaller species of Dictyostelium. Thus, there can be no doubt of its true relationship to the Acrasieae. Only in the terminal process of fructification does it differ basically from other genera already known, However, the ability of this slime mould to construct a supportive stalk without the apparent expenditure of any member cells represents such a major departure from the usual sorocarp building processes seen in the Acrasieae as to necessitate the establishment of a new genus to include it.

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* Harper introduced the term sorocarp for the mature fructification and sorophore for the supporting stalk in Dictyostelium (1926). In his study of Polysphondylium (1929) sorogen was used for the mass of undifferentiated myxamoebae of the completed fructification. These usages, together with sorus for the mass of differentiated spores (Zopf, 1885; Olive, 1901, 1902), have become generally adopted by students of the Acrasieae and will be used here.

The binomial *Acytostelium leptosomum*, proposed in recognition of its acellular sorophores and the generally diminutive proportions of its sorocarps, was first applied to the new genus and species in a paper published earlier (Raper, 1956*b*). The name was introduced to permit a brief comparison of the morphogenesis of this slime mould with that of other genera and species already known. It was not then possible to present an extended account or to include adequate technical descriptions of the new genus and species; nevertheless, a brief Latin description was published with accompanying photomicrographs to validate the use of the name. Much additional study has since been devoted to this unique member of the Acrasieae, and it is now possible to present full technical descriptions of the genus and species, together with accounts of its isolation, its cultivation, and the more salient features of its developmental history.

The presence of a cellular (Dictyo = net-like) stalk is so important a character of the Dictyosteliaceae that recognition of a new family, the Acytosteliaceae, is required also. However, since the new family would at present embrace only the single genus *Acytostelium*, the specifications for it would be identical with those of the genus, which are presented herewith:

TECHNICAL DESCRIPTIONS

Acytostelium Raper, nov.gen.*

In vegetativo statu, myxamoebae liberae bacteria edentes; in fructificativo statu, typica aggregatio myxamoebarum in radiatis, variis dimensionibus, pseudoplasmodiis et in subsequentibus sorocarpis cum acellularibus sorophoris terminales soros protantibus.

Vegetative stage consisting of free-living, independent myxamoebae which feed upon bacterial cells; fruiting stage characterized by the aggregation of such cells to form radiate pseudoplasmodia of varying dimensions and the subsequent development from these of sorocarps composed of acellular sorophores bearing masses of spores as terminal sori.

Acytostelium leptosomum Raper, nov.gen. et sp.*

Cultum ad 22–27° in solido 'glucose + peptone' substrato (agar-agar) cum bacteriis, *Aerobacter aerogenes* vel *Escherichia coli*: myxamoebae vegetativae, liberae, uninucleatae, forma et dimensionibus variis, cum acutis pseudopodiis; pseudoplasmodia typice radiata, dimensionibus linearibus multum variis, sorocarpi gregarii, maxime delicati, cum erectis sorophoris, acellularibus, non ramificatis, plerumque 750–1500 μ. longis, 1·0–2·0 μ. diam., cum terminalibus soris; sori globosi, plerumque 30–50 μ. diam.; sori sphaerici, plerumque 5·0–7·0 μ. diam.

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* *Mycologia* (1956), 48, p. 179, figs. 4 and 6.

Hab. Ex mucidis foliis in superiore solo, per deciduas silvas, isolatum, Illinois, Michigan et Wisconsin, U.S.A. (Typus isolatus: FG–12 a, Junio 1952).

Cultures grown upon dilute glucose + peptone or lactose + peptone agars in association with *Aerobacter aerogenes* or *Escherichia coli* at temperatures between 22 and 27°: vegetative myxamoebae free-living, uninucleate, with pointed pseudopodia, variable in form and size, commonly 12–18 μ. × 7·5–10 μ.; pseudoplasmodia typically radiate, varying greatly in linear dimensions; sorocarps gregarious, extremely delicate, consisting of erect, unbranched, acellular sorophores, commonly 750–1500 μ. long by 1·0–2·0 μ. diameter, bearing terminal sori; sori globose, mostly 30–50 μ. diameter; spores spherical commonly 5·0–7·0 μ. diameter. Myxamoebae often encysting as individuals to form microcysts.

Habitat. Isolated from leaf mould and surface soil from deciduous forests, Illinois, Michigan, Wisconsin, U.S.A. (Type strain FG–12 a, isolated in June 1952).

ISOLATION AND CULTIVATION

Acytostelium leptosomum was first isolated in 1952 by the senior investigator and Miss Dorothy Fennell from leaf mould collected from a virginal deciduous forest of oaks, hickory, maple and associated trees and shrubs at Funk's Grove, Illinois. The slime mould was isolated by our conventional technique of streaking an aqueous suspension of the macerated source material on hay- and thin hay-infusion agar plates (Raper, 1937, 1951), allowing the slime moulds to fruit, and then transplanting spores from the more isolated sori to fresh agar plates previously cross-streaked with *Escherichia coli*. We did not at the outset realize that we were isolating a new and unique slime mould, but instead suspected it of being an unusually diminutive *Dictyostelium minutum*.

Because of its small dimensions and the ephemeral nature of its sorocarps, *Acytostelium leptosomum* was with difficulty obtained in two-membered culture with various bacterial associates. Of these, *Escherichia coli*, generally used for other species, is satisfactory when careful attention is given to the composition of the substrate. The red-pigmented species *Serratia marcescens* can also be used with the resulting sorocarps developing a slight pinkish coloration suggestive of that seen more intensified in *Dictyostelium discoideum* and in *Polysphondylium violaceum* (Raper, 1937, 1951). The most generally satisfactory associate seemed to be *Aerobacter aerogenes*, and the new slime mould has been grown with this Gram-negative bacterium more often than with any other.

The slime mould may be grown by spotting or streaking a mixed inoculum of nutritive bacteria and spores on a suitable culture medium. However, we have generally pre-planted agar plates of the selected substrate with bacteria, either in the form of crossed streaks or as broad bands (*c.* 3·0 cm. wide), and subsequently added spores of the slime mould at the intersection of the crossed streaks or at 3 or 4 evenly spaced loci within the bands of bacterial growth in the other type of culture. In plates of the latter type the slime mould initiates growth at the points of inoculation and spreads radially from these, the resulting colonies attaining diameters of 3–4·5 cm. within 7–10 days. Radiate pseudoplasmodia typical of the Acrasieae normally appear at these centres after 3–4 days (Pl. 1, fig. 2) and others develop progressively outward from them as the frontiers of feeding myxamoebae advance (Pl. 1, fig. 1). Beautifully proportioned sorocarps generally develop from the first-formed pseudoplasmodia; those arising from aggregates formed subsequently commonly appear atypical or abortive. Not infrequently, sorocarps never develop from the aggregates which arise after the culture is 5–6 days old, presumably as a consequence of the accumulation of inimical but as yet unidentified products of the 'host's' metabolism.

Subsequent to the isolation of the original and type strain of *Acytostelium leptosomum*, no. FG–12a, cultures have been twice obtained from comparable leaf mould samples collected from deciduous forests in the Arboretum, University of Wisconsin, and once from the area of Gull Lake, Michigan. These appear to be like no. FG-12a in general dimensions and growth response on different substrata.

Acytostelium leptosomum has been cultivated upon a variety of substrates in two-membered cultures with *Aerobacter aerogenes* and with *Escherichia coli*. Results to date have been rather inconsistent, and it is difficult to recommend any single combination of culture medium and nutritive bacterium as optimal for its growth and fructification. The successful propagation of this slime mould, like that of all other members of the Acrasieae, is dependent upon the realization of a favourable cultural environment to which the composition of the substrate, the fermentative capacities of the bacterial associate, and the physical environment of temperature, pH, etc., all make their separate and conjoint contributions—either favourable or unfavourable. However, two general conclusions can be drawn: *Aerobacter aerogenes* is generally a more satisfactory bacterial associate than *Escherichia coli*, and media of relatively low nutrient content are more productive of normal fructifications than more concentrated media of the types commonly employed for the cultivation of larger members of the Acrasieae (Raper, 1951). Present evidence would seem to indicate that the new genus and species is singularly sensitive to the cultural environment, this often varying in different areas of the same culture dish. We have not yet succeeded in obtaining a truly rich culture of this slime mould in which optimally proportioned sorocarps were abundantly produced.

Culture media. Many agar media containing low concentrations of lactose or glucose and peptone (Difco) or yeast extract (Difco) were investigated. Since it early became evident that the fructification of *Acytostelium leptosomum* was favoured by a mildly acid reaction, the carbon source was added generally at concentrations from 2 to 10 times (w/w) that of the peptone or yeast extract to accentuate this condition. The compositions of some of the more favourable media, solidified with 1·5 % (w/v) agar (Difco), were:

(1) 0·1 % (w/v) lactose + 0·1 % (w/v) peptone. This medium was one of the first used and has served as an arbitrary standard for comparing other media. Colonies initiated as point inoculations in freshly implanted bands of bacterial

growth attained a diameter of 1·5–2·0 cm. within 7–10 days. Small pseudoplasmodia were formed in the central area and from these a limited number of well-proportioned sorocarps normally developed. Fruiting organizations seldom developed in marginal colony areas.

(2) 0·5 % (w/v) lactose + 0·05 % (w/v) peptone. This was a generally satisfactory medium; it supported fairly heavy growth and usually normal fructification. Colonies attained a diameter of 2·0–2·5 cm. within 10 days and were marked by optimal fruiting in central areas, but with either normal or abnormal sorocarps developing outwards from these in localized situations. Microcysts were often produced in great abundance after one week.

(3) 0·5 % (w/v) lactose + 0·025 % (w/v) peptone. Results were much like those with the preceding except that bacterial and slime mould growth was less. Extensive non-fruiting pseudoplasmodia often developed secondarily to smaller primary fruiting aggregates. Microcyst formation was common.

(4) 0·1 % (w/v) lactose + 0·05 % (w/v) yeast extract. Total growth of bacteria and of slime mould was somewhat heavier than on the preceding medium; colonies reached a diameter 3·0–3·5 cm. in 10 days and were marked by optimal fruiting in the central areas, and by extensive pseudoplasmodia up to 1·5 cm. in diameter nearer their margins.

(5) 0·05 % (w/v) lactose + 0·025 % (w/v) yeast extract. Growth of the bacteria and of the slime mould was limited, being less than on medium no. 4. Colonies of the slime mould attained a diameter of 3·5–4·0 cm. in 10 days. Excellent fruiting generally occurred in central areas 1·0–1·5 cm. in diameter, while outward from these extensive non-fruiting pseudoplasmodia often developed. Many of the illustrations for this paper were made from cultures of this type.

(6) 0·5 % (w/v) glucose + 0·1 % (w/v) peptone. Maximum growth of the slime mould and generally satisfactory fruiting was obtained on this medium. Within 10–14 days colonies attained a diameter of 4·5–5·0 cm., reaching the margin of the bacterial growth where massed sorocarps were produced. The latter were often suboptimal in proportions. Microcyst formation was sometimes very extensive and occurred in areas alternating with others wherein abundant sorocarps developed.

(7) 0·05 (w/v) glucose + 0·025 % (w/v) yeast extract. Slime mould colonies were commonly 4·0–5·0 cm. diameter within 10–14 days with good sorocarp formation in central areas 1·0–2·0 cm. in diameter; extensive non-fruiting pseudoplasmodia often developed in areas beyond these.

In addition to the above media, a dilute hay-infusion medium (thin-hay agar; Raper, 1951) was used successfully for the cultivation of *Acytostelium leptosomum* in association with *Aerobacter aerogenes* or *Escherichia coli*, *Serratia marcescens*, *Pseudomonas fluorescens*, *Achromobacter* sp. The amount of bacterial and slime mould growth was comparable to that obtained on medium no. 3 above. Colonies attained a diameter of 4·0–5·0 cm. within 10 days, becoming confluent in the type of cultures employed. Well-proportioned sorocarps developed generally in central colony areas and at isolated islands outward from these. Relatively large non-fruiting pseudoplasmodia sometimes developed, but these rarely attained the dimensions cited above. Altogether, this was a favourable growth medium and it permitted fructification of the slime mould with a wider variety of bacterial associates than any other yet employed. However, its composition is unknown and not reproducible, and the brownish colour always associated with it interferes with microscopic observations. Except for initial isolations it was used less often than the compounded colourless agars listed above.

Hydrogen-ion concentration. It is not possible to cite a narrow and specific range of pH values as optimal for the growth and fructification of *Acytostelium leptosomum* since we have observed abundant, well-proportioned sorocarps upon substrates ranging from pH 4·8 to 6·8. As with other members of the Acrasieae, the pH range that permits myxamoebae to aggregate is slightly wider, and limited growth occurs from approximately pH 4·5 to 7·6. The values listed are, for the most part, those prevailing in unbuffered cultures at 6–10 days. Preliminary studies support the view that Acytostelium is not especially sensitive to pH, and that the most favourable range is *c.* pH 5·5–6·0.

Temperature. The optimal temperature for growth and sorocarp formation in *Acytostelium leptosomum* is approximately 25°. Growth may or may not take place at 30° which appears to be about the maximum temperature tolerated. At 20° growth and fructification was good, but both phenomena proceeded at slower rates than at 25°. At 15° growth was very slow, the colonies seldom attaining a diameter greater than 1·0–1·5 cm. within 10 days. Few pseudoplasmodia developed and from these only a limited number of small sorocarps were produced. Microcysts were regularly produced in great abundance, possibly due to the unfavourable physical environment.

GROWTH AND MORPHOGENESIS

Spore germination

The spores of *Acytostelium leptosomum* are typically spherical (Pl. 1, figs. 4, 5) and resemble those of *Dictyostelium lacteum* van Tieghem (1880), but differ from the capsule-shaped to reniform spores regularly seen in other species of Dictyostelium and in Polysphondylium. They vary considerably in size, ranging mostly from 5·0 to 6·5 μ. diameter, but with individual spores from 4·5 to 7·5 μ. diameter. When compacted within the sorus they appear somewhat polygonal, but when viewed singly in an aqueous mount this aspect is no longer apparent. They possess a thin cellulose wall, and in germination do not release their myxamoebae by a splitting of the spore case as in most species of Dictyostelium and Polysphondylium but rather by the apparent dissolution of an area of the spore wall concurrent with the swelling of the spore content. Following germination, the empty spore case remains as a very delicate hyaline structure that exhibits no evident birefringence.

Vegetative growth

Upon germination the emergent myxamoeba begins to feed upon bacterial cells and divides. The vegetative phase of *Acytostelium leptosomum* thus begun consists, as in all of the Acrasieae, of the separate and independent growth of free-living myxamoebae which increase in numbers by repeated divisions until a large population is built up and the available food supply is exhausted. As this occurs, the fruiting stage is initiated by the aggregation of the previously unoriented myxamoebae into pseudoplasmodia, from each of which several minute sorocarps subsequently develop. The vegetative myxamoebae of the new genus and species (Pl. 2, fig. 10) exhibit no structural or behavioural characteristics that distinguish them from those of a species of Dictyostelium or of Polysphondylium. They are inconstant in form, ranging from irregularly elongate, through wedge-shaped and broadly triangular to more or less rounded. Pseudopodia are filose rather than lobose, and these seldom exceed in length more than one-quarter the overall diameter of the myxamoeba. Whereas these may occur at any position on the surface of the amoeboid cell, they are understandably most prominent at the advancing edge of cells in active motion. The vegetative myxamoebae vary appreciably in mass, and differences in linear dimensions are often pronounced. Whereas the latter must be interpreted with considerable caution, they are of comparative value, and in *A. leptosomum* these range from 18–20 × 8·0–6·5 μ. in the more elongate cells, from 12–16 × 12–8 μ. in those which are expanded and roughly triangular, and from 8·5 to 12·5 μ. in individuals that are more nearly round. Each vegetative cell contains a single nucleus which is difficultly discernible in the living state, and this is normally central within the endoplasm. The actively motile cell exhibits a marginal zone of clear, non-granular ectoplasm most pronounced at the anterior edge, and the small filopodia represent irregular extensions of this material. The main body of the vegetative myxamoeba is composed of a finely granular endoplasm which contains, in addition to the nucleus, food vacuoles enclosing bacteria in various stages of digestion and one or more excretory vacuoles. When present in numbers greater than one, the excretory vacuoles usually coalesce before being discharged in a rhythmic pattern which may vary from cell to cell.

Under optimal cultural conditions the myxamoebae characteristically enter a pseudoplasmodium at the close of the vegetative stage and subsequently contribute to the formation of one of the several sorocarps which rise from it. Not infrequently, however, particularly in older and less favourable cultures, the vegetative cells enter a resting stage as individuals, forming microcysts (Blaskovics & Raper, 1957) by the deposition of thin protective cellulose walls. In extreme cases, under conditions not at present understood, almost all the myxamoebae become so encysted, producing a broad and virtually continuous layer of rounded inactive cells which never become parts of any fruiting organization. In other instances (see later), very large pseudoplasmodia may develop which do not proceed to sorocarp formation, and in these the constituent myxamoebae may in time form microcysts. Whereas the microcysts of most cellular slime moulds (e.g. *Polysphondylium pallidum* Olive and *Dictyostelium polycephalum* Raper as reported by Blaskovics & Raper; see above) differ markedly in appearance from true spores, those of *Acytostelium leptosomum* do not exhibit this distinction. The microcysts, like the spores, are spherical.

Considered as a cell population, however, they average slightly larger than the spores. The dimensions of individual cells of the two types overlap throughout most of their separate ranges, and in a mixed population it is difficult to distinguish between them. Occasionally much larger spheroid to ovoid cells ranging up to 12–15 μ. in diameter are observed in older cultures; the origin and significance of these are not presently known. The walls of the microcysts appear light purple or bluish when stained with chloroiodide of zinc, and when then viewed with polarized light exhibit a marked birefringence, possibly less intense than that of the spores. In *A. leptosomum* the microcyst appears to germinate in the same manner as true spores and the released myxamoeba leaves behind a thin, hyaline case from which apparently a portion of the wall has been dissolved.

Pseudoplasmodia

At the end of the vegetative stage, which is normally coincident with the exhaustion of the food supply (Pl. 2, fig. 6), the previously independent and unoriented myxamoebae become conspicuously elongate in the direction of emergent centres toward which they aggregate by their co-ordinated movements, probably prompted and controlled by a diffusible chemotactic agent comparable to that reported for *Dictyostelium discoideum* (Bonner, 1947 *et seq.*; Shaffer, 1953, 1956*a*, *b*; 1957; Sussman, Lee & Kerr, 1956). As this process continues the inflowing myxamoebae collect into progressively larger converging streams (Pl. 2, figs. 8, 9) producing radiate pseudoplasmodia (Pl. 1, figs. 1, 2) basically similar in pattern and structure to the wheel-like aggregates formed in species of Dictyostelium and Polysphondylium. The degree of cell elongation during the aggregative process varies in different pseudoplasmodia and within different streams and areas of the same organization. To an appreciable extent the degree of such elongation may be correlated with the rates of movement of individual cells; in the more actively streaming myxamoebae the length of a cell is not uncommonly 5–6 times its greatest diameter, dimensions of 25–30 × 5–6 μ. being common.

The pseudoplasmodia of *Acytostelium leptosomum* vary greatly in size, ranging from less than 1 mm. to several cm. in diameter. There is reason to believe that the latter (Pl. 2, fig. 11) represent a somewhat abnormal development, for such expansive organizations generally form in marginal areas of older colonies after smaller aggregates have developed near the sites of inoculation. Furthermore, the large pseudoplasmodia seldom give rise to normal sorocarps, whereas the smaller, first-formed aggregates characteristically produce these in varying numbers up to twenty-five or more (Pl. 1, fig. 3). Not uncommonly, the very large organizations never progress beyond the stage of cell aggregation, and in time the constituent myxamoebae may form microcysts as already described or they may eventually disintegrate *in situ*.

Under optimal conditions, and in cultures initiated by point inoculations, pseudoplasmodia begin to develop within 3 days from the time of implantation and may continue to form for 7 days or more thereafter, being removed 2 or 3 days behind the advancing front of vegetative myxamoebae (Pl. 2, fig. 6).

The first-formed aggregates are always radially symmetrical in pattern, whereas those subsequently developed assume the outlines dictated by the areas from which the contributory myxamoebae are drawn. As aggregation proceeds, and often many hours before the process is complete, numerous minute papillae appear on the surface of the central, slightly elevated portion of the pseudoplasmodium, and in each of these the construction of a sorocarp is soon begun. To a degree seldom observed in other members of the Acrasieae, aggregation and culmination proceed simultaneously within the same overall community of cells (Pl. 2, fig. 7). The events taking place in any particular part of this community obviously reflect the stage of maturity currently attained by the cells of that area. It is suggested that a chemotactic stimulus capable of attracting the myxamoebae initially may prove inadequate to direct further integration and differentiation essential to sorocarp formation. On the other hand, quite different regulatory substances may be operative in aggregation and in culmination.

We have at present no satisfactory explanation for the formation of the very large and virtually centreless pseudoplasmodia in *Acytostelium leptosomum* (Pl. 2, fig. 11), which often persist for days. On the one hand they may originate from a definite and seemingly normal primary centre from which very long, rebranched, and more or less anastomosing streams extend outward for 1 cm. or more (Pl. 2, fig. 11). More commonly, no definite centre can be distinguished and the component streams seem merely to focus on a general area adjacent to sites where earlier and more nearly normal pseudoplasmodia were formed. Not infrequently, only a general area of attraction can be discerned and streams simply originate and disappear in areas where equally dense populations of myxamoebae remain unaggregated and unoriented. For the former case it was suggested (Raper, 1956*b*) that an Acytostelium-acrasin is produced, and that this stimulates the myxamoebae to aggregate in characteristic fashion, after which the stimulus fades and the cells are left leaderless—yet they do not disperse. For the latter cases, where no definable centres are obvious, an alternative explanation might suggest that extensive fields of myxamoebae may arrive almost simultaneously at a receptive stage, but the points of Acytostelium-acrasin production are so numerous as to preclude the ascendance of any well-defined centres. In any case, we are reasonably certain that the formation of these large aggregates reflects a measure of cultural adversity.

Sorocarps

The uniqueness of *Acytostelium leptosomum* becomes apparent only during the process of sorocarp formation or culmination, as Bonner (1944) designated this terminal phase of development. Whereas the pseudoplasmodial stage possesses certain distinctive features, particularly the very large non-fruiting aggregates, even these are not regarded as basically dissimilar to the pseudoplasmodia of other genera. The continuing influx of late-arriving cells concurrently with the differentiation into sorocarps of others previously assembled is most striking in this slime mould, yet these same phenomena are seen to overlap in varying degrees in almost all members of the Acrasieae. This is particularly true in two of the smaller species of the genus *Dictyostelium*, *D. lacteum* van Tieghem (1880) and *D. minutum* Raper (1941). Furthermore, a gregarious habit comparable with that of *Acytostelium leptosomum* is regularly observed in these slime moulds also, differing from the new genus and species principally in the production of fewer sorocarps from a single primary pseudoplasmodium. Thus, no developments occur in *A. leptosomum* before the actual construction of the sorocarp, which would necessitate the creation of a new genus.

The completed sorocarp of *Acytostelium leptosomum* consists of a very delicate unbranched tubular sorophore supporting a globose sorus at its terminus. The dimensions of such fructifications vary in different cultures and in different areas of the same culture, depending apparently upon localized environmental conditions. However, their dimensions never exceed certain maxima which we believe to reflect the greatest number of myxamoebae that can collaborate successfully in sorocarp formation. In well-proportioned sorocarps the sorophores commonly range between 750 and 1500 μ. in length by 1·0–2·0 μ. in diameter, although shorter structures are commonly observed and others up to 1·8 mm. in length are occasionally seen. Sorophore diameters seldom fall outside the stated range, even in structures that are obviously abnormal. The diameter of the sori commonly range between 20 and 50 μ. but smaller spore masses are often seen and an occasional sorus may reach 60 μ. diameter. As in species of Dictyostelium and Polysphondylium, the sorus consists of a mass of spores suspended in cohesive slime which maintains its globular form.

The sorophore of *Acytostelium leptosomum* is of special interest since it is acellular and must, therefore, represent a secreted structure in its entirety. This is in marked contrast to the sorophore of the Dictyosteliaceae which consists of strongly vacuolated and compacted cells which differentiate within a previously secreted tubular 'mold' termed the sorophore sheath. This sheath of cellulose is deposited extracellularly at the appropriate circular locus within the developing sorocarp, or sorogen, by a continually changing group of anteriorly placed myxamoebae*. In *Acytostelium leptosomum* the stalk itself is believed to be of comparable origin, but with the important difference that the secreted cellulose is deposited by anteriorly placed myxamoebae into an extremely narrow intracellular trough centrally positioned in the apical portion of the sorogen. Because of this, the lumen of the sorophore is so small as to preclude the entrance or entrapment therein of any myxamoebae and so all of the cell population is available for the production of spores. There can be little question but that this represents, in many ways, a distinct advance over Dictyostelium and Polysphondylium where a substantial fraction (*c.* 20–25 % in *Dictyostelium discoideum*) of the total population is sacrificed in the construction of supportive tissue, namely the sorophore. On the other hand, the new genus is possibly handicapped by this very process of cell conservation, for it probably limits the dimensions to which the sorophores and sorocarps can be constructed.

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* For details of sorocarp formation in Dictyostelium see Raper & Fennell (1952) and Bonner *et al.* (1955).

The first indication of sorocarp formation is the appearance of minute papillae upon the upper surface of the central area of the aggregating pseudoplasmodium (Pl. 3, fig. 14). Such incipient sorogens increase in height and soon may be observed to be free of the main body of inflowing myxameobae and to be supported on very delicate stalks (Pl. 2, fig. 7; Pl. 3, figs. 12, 13). By this time the separate masses of fructifying myxamoebae have assumed an irregularly fusiform shape, which with minor variations is generally maintained until sorophore formation is almost completed. Figures 12 and 13 (Pl. 3) and fig. 20 (Pl. 4) illustrate this point; fig. 15 (Pl. 3), on the other hand, shows a sorogen shortly before the beginning of spore maturation in which the apical portion is more nearly rounded. An interesting feature of all these stages, and one that can be detected quite early (Pl. 3, fig. 14), is the transverse orientation of the myxamoebae which comprise the sorogens. This condition is most clearly seen in figs. 20 and 15, and it poses some very intriguing problems concerning the mechanics of sorocarp building in this singular slime mould.

In their study of sorocarp formation in Dictyostelium, Raper & Fennell (1952) pointed out that cells oriented in a transverse direction, such as the myxamoebae comprising the apical tip of the developing sorocarp of *Dictyostelium discoideum*, could not contribute effectively to the lifting force responsible for raising the sorogen. It was concluded that this force had its origin in the vacuolating (and hence enlarging) cells entrapped within the lengthening sorophore sheath, and that its effectiveness stemmed from the exertion of substantial pressure against the cells directly above the open end of the sorophore sheath and through them upon the slime covering that continuously enveloped the apical area until the completion of culmination. In *D. discoideum* the remainder of the sorogen, including the mass of differentiating spores of later stages, simply adhered to the apical portion by cohesive forces and advanced with it. The lengthening sorophore of *Actyostelium leptosomum* contains no vacuolating cells; therefore, the effective lifting force must be sought elsewhere, and this cannot reasonably be considered to arise from the transversely oriented myxamoebae (Pl. 4, fig. 20). The following hypothesis is tentatively advanced: through the co-ordinated behaviour of the anteriorly positioned myxamoebae a very narrow and vertically elongate intracellular channel is maintained centrally in the apical portion of the sorogen. Into this space the surrounding cells secrete cellulose (or its precursors), while the continuous accumulation of this substance anteriorly, coupled with the centripetal restraining force exerted laterally by the closely packed cells, results in a forward pressure which is exerted upon the slime encased cells that close the channel at its anterior end. Thus the tip is advanced and, as in Dictyostelium, the remainder of the body follows, being drawn forward by cohesive forces. It is believed significant that in *A. leptosomum* a sorogen with an apical tip of sharply reduced diameter such as that seen in Dictyostelium has not been observed in a developing sorocarp. Apparently, spore maturation occurs rapidly, and present information would indicate that this process is not initiated until the formation of the sorophore has been completed. Thus the entire sorogen in this new genus may act during culmination in a manner comparable with the apical tip of a developing sorocarp of Dictyostelium, except that it loses none of its cellular members in constructing the sorophore.

There is, we believe, substantial support for the foregoing hypothesis. Plate 4, fig. 20 shows quite clearly the characteristic transverse orientation of the myxamoebae in a developing sorocarp. The accompanying illustration (fig. 21), photographed with polarized light, shows equally well the extension of the delicate sorophore far into the anterior third of the sorogen. Figures 22 and 23 (Pl. 5) illustrate the close horizontal compacting of the myxamoebae in this portion of the sorogen and also the effective closure anteriorly of any hypothetical channel within this area by strategically positioned terminal myxamoebae, or 'cap cells'. In figs. 24 and 25 (Pl. 5) the horizontally flattened, wedge-shaped cells of the apical region are seen dissociated, while in fig. 26 a small circular segment of the same region shows such cells in their normal positions as viewed vertically from the bottom or the top (?) of the sorogen. Significantly, this segment shows a minute opening at its centre which we think could represent the postulated open secretory channel seen in cross section.

The extremely delicate nature of the sorophore in proportion to the size of the spore mass that it supports is well illustrated in Pl. 1, fig. 4, while in the adjacent photomicrograph (fig. 5) this is re-emphasized by showing its diameter to be only a fractional part of one of the hundreds of spores that it supports. Figure 16 (Pl. 3) shows some of these structures bent to reveal their tube-like nature. Figure 17 shows the base of one of these, here protrayed as broadening and thinning downward; but, as seen *in situ*, this portion of the stalk is expanded radially on the agar in the pattern of a flaring skirt and gives the sorophore a considerable measure of vertical support. Finally, the cellulosic nature of the sorophores, and of the spore walls as well, is substantiated by figs. 18 and 19 (Pl. 4), where a preparation was treated with chloroiodide of zinc and subsequently photographed under normal and polarized light.

Considering the fact that the erect sorocarps of *Acytostelium leptosomum* are apparently never branched, one might surmise that the myxamoebae which constitute a rising sorogen are committed to the formation of one and only one fruiting structure. Such is not the case. If the sorogen of an incompleted sorocarp topples over and touches the agar surface sorophore formation ceases forthwith, the mass of undifferentiated myxamoebae reorganizes into one or more secondary fruiting organizations, and a small but thoroughly normal sorocarp subsequently arises from each of the fractional parts. In one example, a sorophore 1200 μ. in length had been constructed prior to such sorogen-to-agar contact, and thereafter two smaller erect sorocarps with sorophores 650 and 525 μ., respectively, developed. In another instance, a sorogen which had already produced a sorophore 1400 μ. in length segmented into four parts following contact with the agar, and from these parts minute sorocarps having sorophores 350, 425, 450 and 525 μ. in length subsequently developed. As one would anticipate, the primary and secondary sorophores were not interconnected. Only in the source of their constitutive cells could the latter sorogens be considered continuous with the first; the intercellular organizations leading to their formation were, of course, new. The behaviour of these fruiting masses is not reported to suggest their uniqueness. Quite the contrary, for when the developing sorocarp of any cellular slime mould is seriously disturbed, adjustments and reorganization of its cellular components regularly follow, the extent of these changes being determined by the severity of the dislocations that prompted them. In the case of *Dictyostelium mucoroides* Brefeld (1869) or *D. purpureum* Olive (1901) mere contact of an intact sorogen with the agar surface would not lead to a comparable reorganization, for such contact commonly occurs and it is *via* this device that the developing sorocarps build long sorophores in the direction of light. On the other hand rupture of the sorogen in these latter species, whether by natural or induced causes, concurrent with agar contact would be followed by the fragmentation of the primary mass into two or more smaller fruiting organizations. The difference in degree of response exhibited by the contrasting genera can undoubtedly be correlated with (1) the thickness and tenacity of the slime envelope that surrounds the sorogens in the two genera, (2) the dimensions of the sorophores in relationship to those of the parent sorogens, and (3) the strengths of the forward building forces generated by the developing sorophores in relationship to the resistance offered by the cell masses resting upon an agar surface. Finally we record that in *Acytostelium leptosomum*, as in species of Dictyostelium, if the constituent cells of a sorogen are disassociated in the presence of nutritive bacteria they will again resume the vegetative phase and fruiting will not recur until the normal cycle has been repeated.

Thus it is seen that *Acytostelium leptosomum* nov.gen. et sp. normally exhibits many of the characteristics common to all of the Acrasieae, and particularly so of those genera that comprise the Dictyosteliaceae. On the other hand, the construction of its sorocarps with their inordinately thin acellular sorophores, however produced, clearly sets the genus and species apart from all previously known members of the Acrasieae and adds new dimensions to earlier concepts of what initially independent cells may achieve through progressive integration and co-ordinated differentiation.

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EXPLANATION OF PLATES

Plate 1

General characteristics of *Acytostelium leptosmum*

Fig. 1. Culture growing on lactose (0·1 %) + yeast extract (0·05 %) agar in association with *Aerobacter aerogenes*; incubation 10 days at 24–26°. Note the clustered sorocarps at the colony centre and the developing pseudoplasmodia in the marginal area. × 12.

Fig. 2. A single psuedoplasmodium showing the typical radiate pattern assumed by the aggregates which develop initially; sorogens are just beginning to form at the dense centre. × 32.

Fig. 3. A cluster of sorocarps in various stages of development arising from a single pseudoplasmodium such as that shown in the preceding figure. × 32. (Reprinted from *Mycologia* (1956), 48, 180.)

Fig. 4. A single sorus with its very thin, unbranched sorophore; the sorus is flattened by the pressure of the coverglass. Preparation stained with erythrosin in aqueous phenol (5 %). × 280.

Fig. 5. Spores stained with erythrosin in aqueous phenol together with two sorophores. × 1300.

Plate 2

Pseudoplasmodium formation in *Acytostelium leptosomum*

Fig. 6. A portion of a colony growing in association with *Aerobacter aerogenes* on lactose (0·1 %) + peptone (0·05 %); incubation 10 days at 24–26°. Note the uninvaded portion of the bacterial streak (upper right), the front of feeding myxamoebae and, in the older portion of the colony, the developing pseudoplasmodia. × 10.

Fig. 7. Two aggregating pseudoplasmodia from which sorocarps are already developing. × 30.

Fig. 8. Peripheral area of an aggregating pseudoplasmodium showing the origin of the inflowing streams of myxamoebae; stained with trypan blue in aqueous phenol (5 %). × 120.

Fig. 9. Details of a portion of an inflowing stream from a pseudoplasmodium like the preceding; note the orientation and elongate form of the myxamoebae. × 625.

Fig. 10. Vegetative, free-living (unoriented) myxamoebae, similarly killed and stained and photographed *in situ*. × 700.

Fig. 11. Large, non-fruiting pseudoplasmodia developed on lactose (0·1 %) + yeast extract (0·05 %) agar in association with *Aerobacter aerogenes*; incubation 10 days at 24–26°. × 8.

Plate 3

Sorocarp formation in *Acytostelium leptosomum*

Fig. 12. A fructifying organization showing the continued aggregation of myxamoebae (lower right) concurrent with the development of several sorocarps. × 80.

Fig. 13. An enlarged view of three of the sorogens seen in the preceding figure; note their characteristic fusiform shape. × 340.

Fig. 14. A very young sorogen at about the beginning stage of sorphore formation; note that the myxamoebae in the terminal area already show evidence of transverse orientation. × 650.

Fig. 15. A sorogen in an advanced stage of sorocarp formation. Note particularly the conspicuous transverse orientation of the myxamoebae, and the rounded outline of the apical portion of the body. × 650.

Fig. 16. Typical sorophores seen in their median areas showing the manner in which they bend, indicating their tube-like structure. × 1280.

Fig. 17. Expanded base of a sorophore. Seen *in situ*, this portion of the stalk is spread out on the agar in the form of a flaring skirt and supports the sorophore in an upright position. × 1280.

PLATE 4

Sorocarp formation in *Acytostelium leptosomum*

Fig. 18. Sorophores and spores stained with chloroiodide of zinc. The sorophores, and to a lesser extent the spores, stain blue with this reagent, indicating their cellulosic nature. × 120.

Fig. 19. The same photographed with polarized light. Note the strong birefringence of the sorophores and the less intense birefringence of the spore walls, further confirming their cellulosic nature. × 120.

Fig. 20. A developing sorocarp. Note the transverse orientation of the constituent myxamoebae and the extension of the sorophore into the apical area. × 825. (Reprinted from *Mycologia* (1956), 48, 197.)

Fig. 21. The same structure photographed under polarized light. Note that the birefringence is evident almost to the terminus of the sorogen. × 825.

PLATE 5

Sorophore formation in *Acytostelium leptosomum*

Fig. 22. Terminus of a sorogen killed and lightly stained with iodine in 70 % alcohol, as seen in surface view without distortion. Note particularly the cell at the apex, termed the 'cap cell'. × 1350.

Fig. 23. Terminus of a sorogen stained with erythrosin in aqueous phenol (5 %) mounted under a coverglass, hence compressed. Note the flattened cells and particularly the 'cap cell' with its nucleus. × 1350.

Fig. 24. Broken terminus of a sorogen stained with trypan blue in aqueous phenol, showing the horizontally flattened, semicircular cells characteristic of this area. × 700.

Fig. 25. Dissociated cells from the subterminal portion of a second sorogen treated in a similar way. Note the wedge-shaped pattern of the cells from this area. × 700.

Fig. 26. A small intact disk of cells from a sub-terminal position at which site sorophore formation takes place. It is suggested that the sorophore is formed by the secretion of cellulose (or its percursors) into a small, centrally positioned lumen, here seen in cross-section. × 700.

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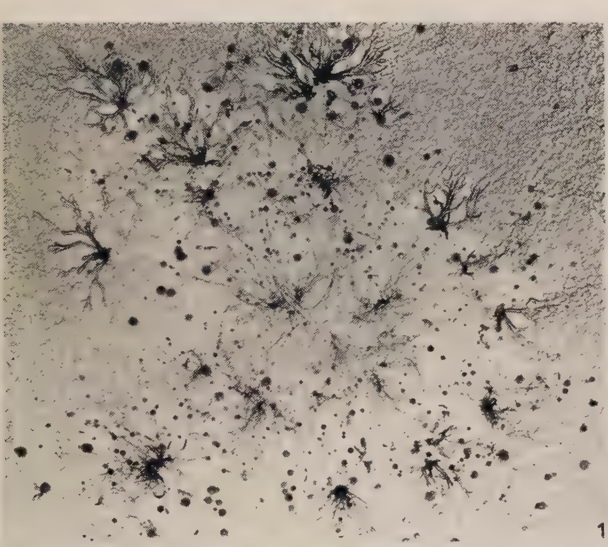

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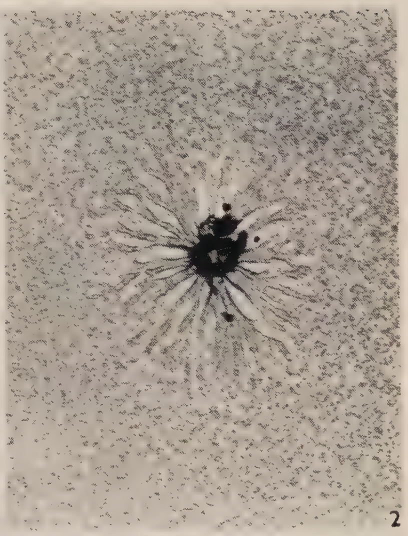

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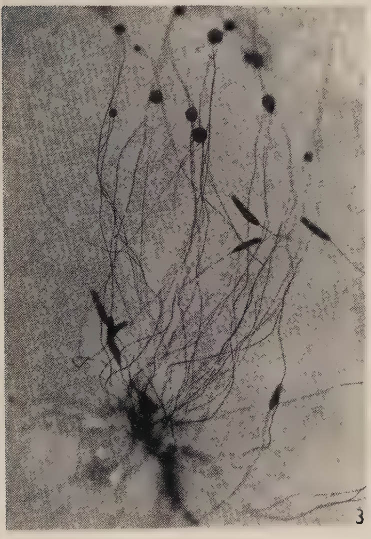

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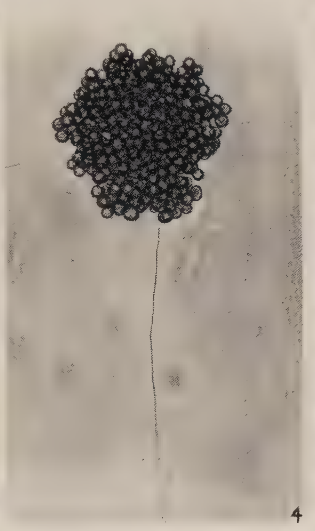

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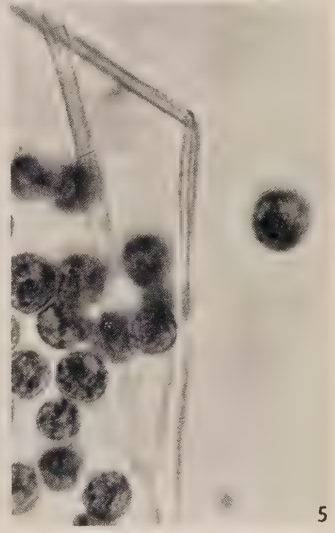

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K. B. RAPER & M. S. QUINLAN—*ACYTOSTELIUM LEPTOSOMUM*. PLATE 1

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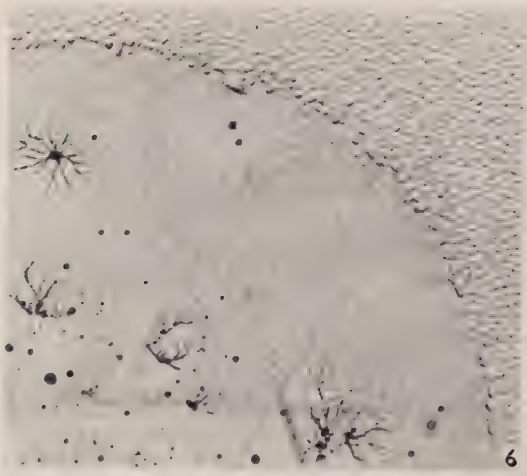
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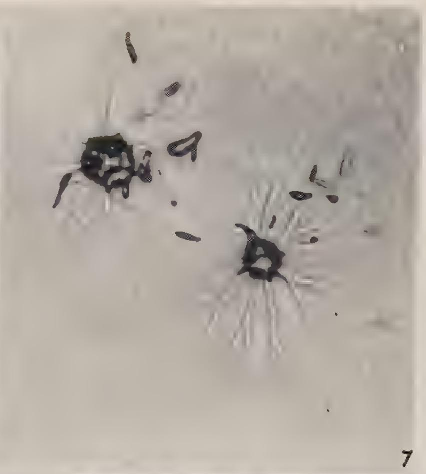
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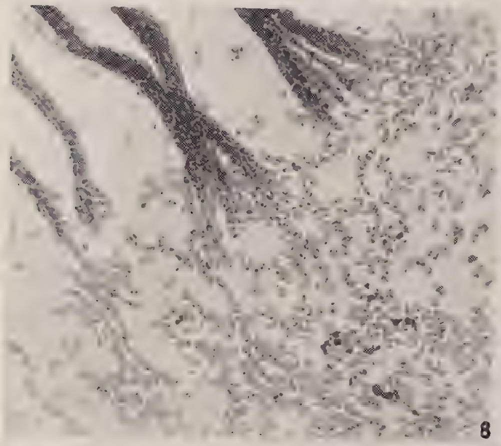
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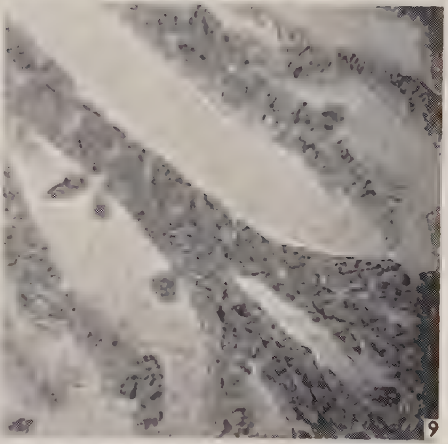
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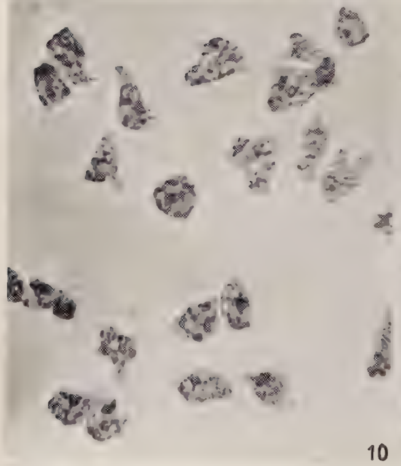
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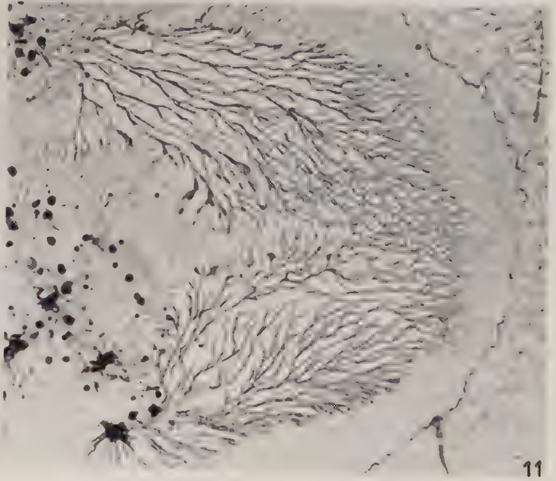
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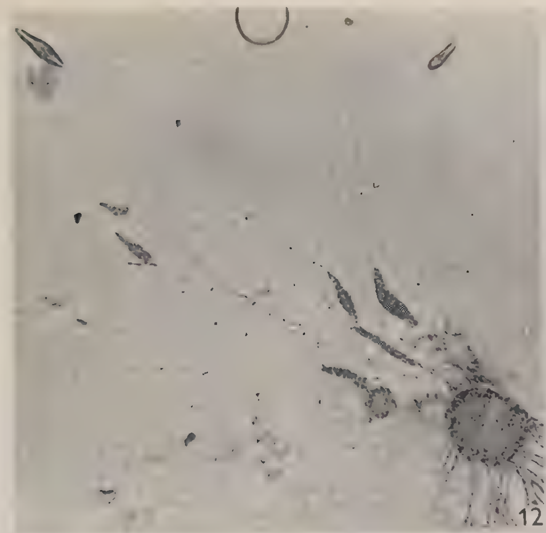
K. B. RAPER & M. S. QUINLAN—*ACYTOSTELIUM LEPTOSOMUM*. PLATE 2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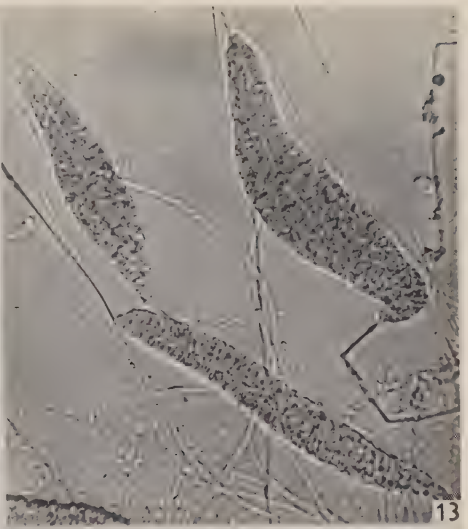
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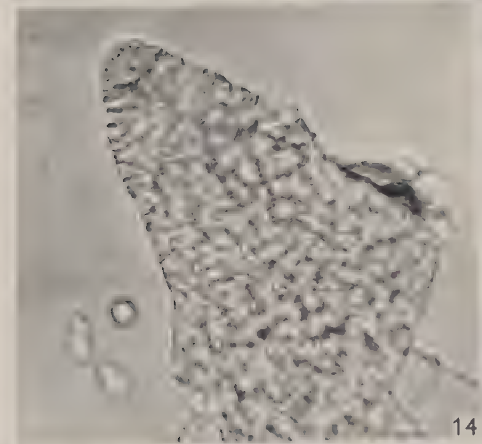
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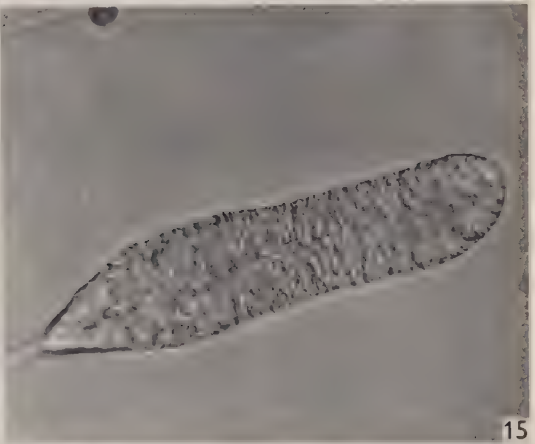
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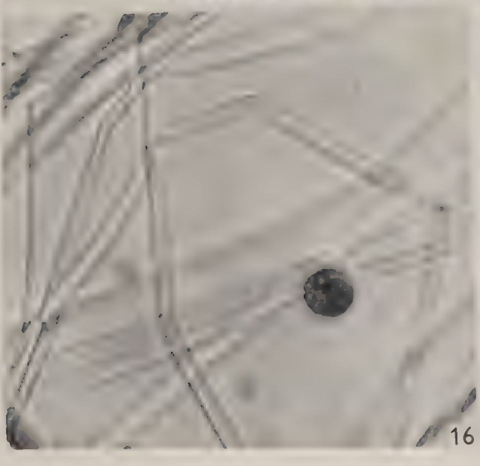
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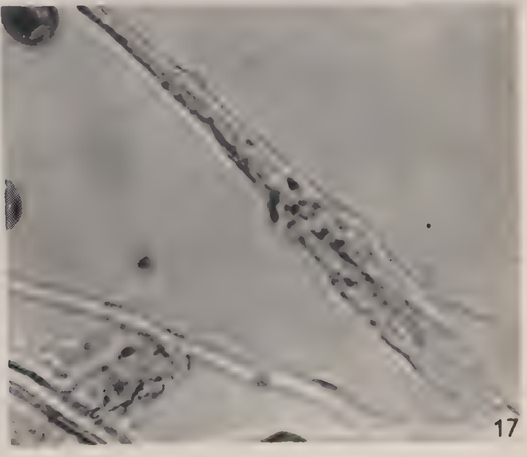
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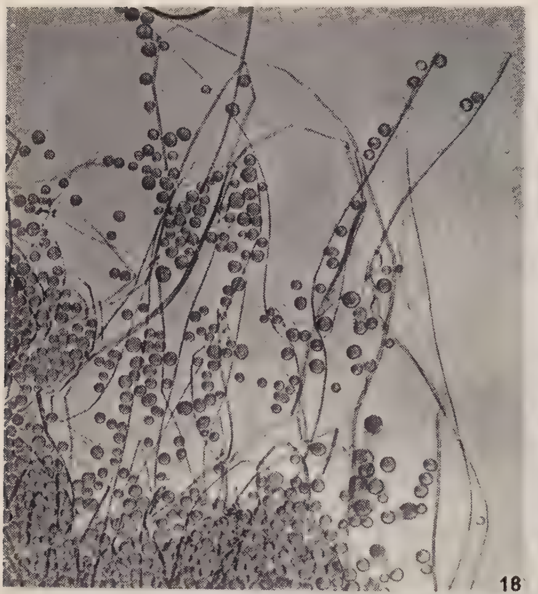
K. B. RAPER & M. S. QUINLAN—*ACYTOSTELIUM LEPTOSOMUM*. PLATE 3



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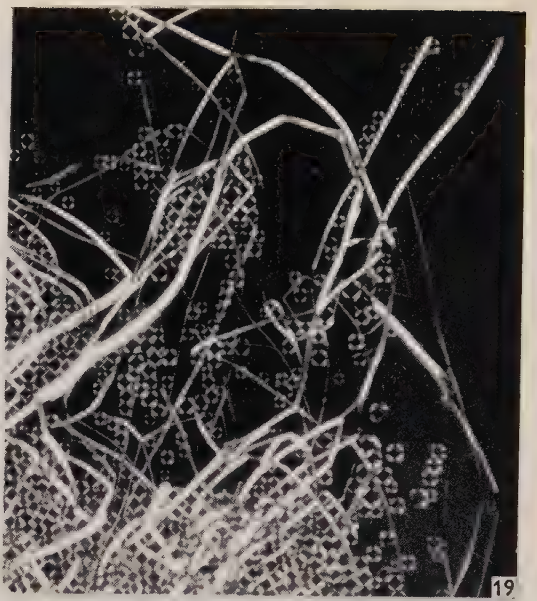

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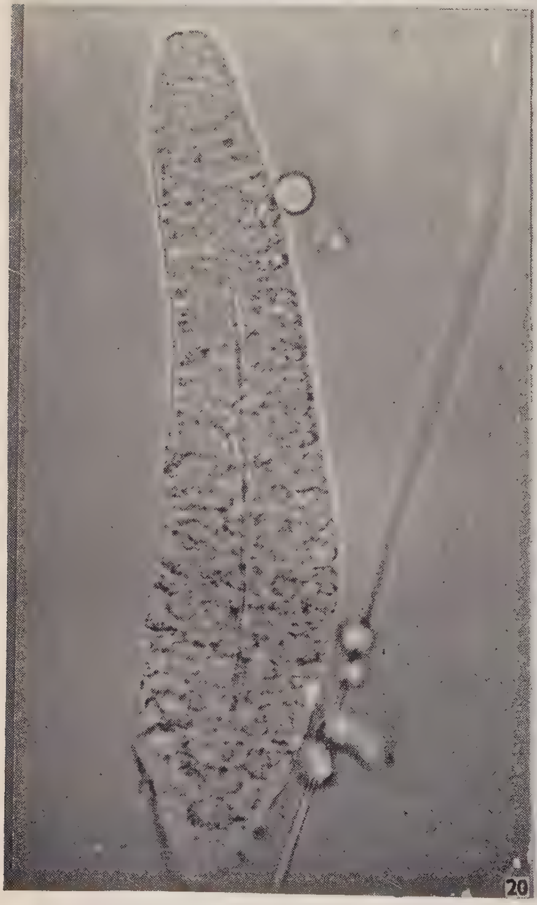

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K. B. Raper & M. S. Quinlan—*Acytostelium leptosomum*. Plate 4

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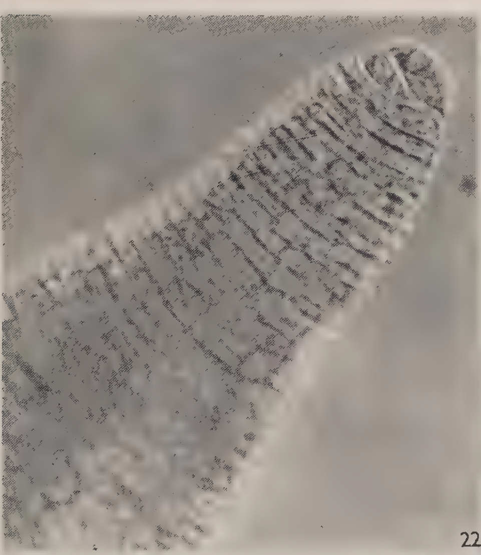
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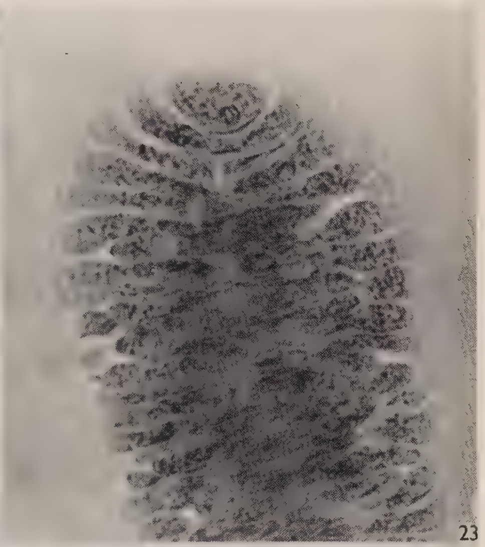
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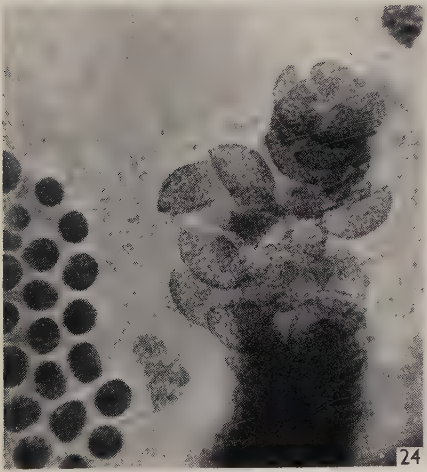
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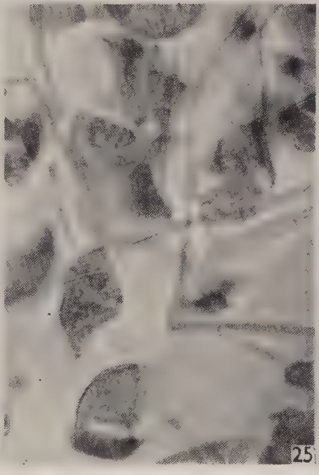
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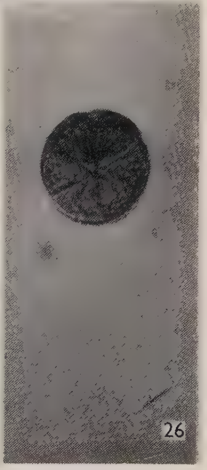
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K. B. Raper & M. S. Quinlan—*Acytostelium leptosomum*. Plate 5

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A Survey of the Production of Pectic Enzymes by Plant Pathogenic and other Bacteria

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SUMMARY: Sixty-seven cultures of plant pathogenic bacteria, of the genera *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas* and *Xanthomonas*, and 56 cultures of non-pathogenic bacteria were tested for production of pectic enzymes in culture. γ-Pectinglycosidase was produced by 22 strains of pathogenic bacteria and by 15 non-pathogens. Pectin methyl esterase was produced by a few pathogens: the soft-rot erwinias, *X. campestris* and one of three strains of *X. vasculorum*.

Bacteria of several genera are known to degrade pectic materials and, in fact, this ability can be used to distinguish *Erwinia carotovora* from the very similar non-phytopathogenic species *Klebsiella aerogenes*. There is, however, very little knowledge of the enzymic processes involved, of how the bacterial enzymes differ from similar plant or fungal enzymes or of the differences between the enzyme systems of pathogenic and non-pathogenic species. Some of the latter degrade pectin with great rapidity and yet are unable to cause a soft-rot of the living plant.

These questions should be resolved for a proper understanding of the disease processes and for the possible development of protective measures based on prevention of pectin breakdown. With this in mind bacteria of several genera have been tested for ability to produce pectic enzymes *in vitro*. The procedures used were designed to give some indication of the character of any pectic enzyme which might be observed.

Pectolytic activity in fungi, which have been studied more extensively than bacteria, has generally been found to depend upon a complex of enzymes which may include pectin methyl esterase (PME) and one or more of a group of glycosidases. The classification and nomenclature of the latter are somewhat confused: many can be fitted into the scheme outlined in Table 1, but this scheme should be regarded as illustrative rather than definitive until further work, with standardized methods, shows which characteristics are intrinsic to the various groups. The testing procedure adopted was planned on the framework of Table 1. Schubert's (1954) term pectin glycosidase (PG) is preferred.

METHODS

General procedure. The bacteria were grown in a complex medium containing pectin. Samples were withdrawn at intervals and tested for the presence of PME and PG and for the degradation of the pectin. To detect α-, β- and γ-PG substrates of high- and low-methoxyl pectin, at pH values of 3·5, 5·5 and 7·5, with appropriate additions of Ca^{++} and NaCl (see Table 2) were used. It was hoped that this range would also reveal any other pectic enzymes that might be produced, but, as a further test for unsuspected enzymes, destruction of the pectin in the culture was tested by the nature of the precipitate formed on adding ethanol.

Table 1. *Enzymes degrading pectic substances*

Names proposed	β-Pectinglycosidase (1), β-polygalacturonase or enzyme I*b* (2)	α-Pectinglycosidase (1), α-polygalacturonase or enzyme I*a* (2)	γ-Pectinglycosidase (1)
pH optimum	*c.* 3·5	*c.* 5·5	>7·0 (1), (3)
Substrate	Pectic acid, low-ester pectin, uronides produced by α-PG and γ-PG	High methoxyl pectin; pectic acid slowly or not at all (1)	High methoxyl pectin, pectic acid (3)
Products	Mono-, di- and tri-galacturonic acid (2)	Uronides not precipitated at pH 1·0, but not mono-, di-, tri-galacturonic acid (2)	Uronides not precipitated by 66 % ethanol
Effects of salts	NaCl contracts and lowers optimum pH range (1)	NaCl spreads and raises optimum pH range (1)	Ca^{++} required for maximum activity (3)
Similar enzymes	*Penicillium expansum* oligase (4), yeast PG (5)	*P. expansum* polyase (4), pectin depolymerase from *Neurospora* sp. (6) polymethylgalacturonase (8)	*Erwinia aroideae* depolymerase (3), enzymes from *Pseudomonas* spp. (7)

(1) Schubert (1954).
(2) Dingle, Reid & Solomons (1953).
(3) Wood (1951).
(4) Ozawa (1951).
(5) Luh & Phaff (1951).
(6) Roboz, Barratt & Tatum (1952).
(7) Oxford (1944).
(8) Seegmiller & Jansen (1952).

Table 2. *Substrates for viscosimetric tests*

Pectic substance:	Pectic acid	Pectin	Pectic acid	Pectin	Pectic acid	Pectin
Concentration (%):	0·5	0·5	0·5	0·5	0·5	0·625
pH value:	3·5		5·5		7·5	
Buffer:	None		0·01 M-succinate		0·01 M-phosphate	
$CaCl_2$:	0		0		0·00125 M	
NaCl (M):	0·2	0	0·2	0·2	0·2	0·2
Phenol (%):	0·2	0·2	0·2	0·2	0·2	0·25

Of the many possible methods of detecting pectolysis of the various substrates, loss of viscosity was chosen because (*a*) it is the most sensitive indicator of degradation of the pectic material, (*b*) it probably reflects the changes significant in soft rotting, (*c*) it can be measured easily and rapidly provided high accuracy is not required. To minimize the effects of substances other than pectic enzymes on the viscosity of the substrates the proportion of culture added was kept low (0·6 ml. + 7·4 ml. substrate). This made an incubation period of 24 hr. necessary and an antiseptic had to be added to prevent microbial growth. Preliminary tests showed that phenol did not affect viscosity diminution by enzymes from *Erwinia carotovora* and 0·2 % (w/v) was incorporated in the substrates.

Organisms. Stock cultures of bacteria, obtained from various sources, were maintained on 'Oxoid' Griffiths Egg-salt Medium (Oxo Ltd., 16 Southwark Bridge Road, London, S.E. 1) stored in the refrigerator after 3–4 days of growth. Designations used by the persons who supplied the cultures were accepted without checking. The nomenclature of *Bergey's Manual* (6th edition) has been used except with the Enterobacteriaceae where the recommendations of the Coli-Aerogenes (1956) Sub-Committee of the Society for Applied Bacteriology have been followed.

When required, subcultures from the egg-salt slopes were grown on slopes of nutrient agar for about 5 days and then transferred to tubes of IM 1 medium (see below) for 2 days before inoculating the flasks of SM 1 medium (see below) in which they were grown for the enzyme tests.

Media. IM 1 medium; Tryptone (Difco), 0·4 %; yeast extract (Difco), 0·01 %, glucose, 0·1 %.

SM 1 medium–solution A; Brown Ribbon pectin,* 5 g.; glucose, 0·5 g.; in 500 ml. water at pH 6·5–7·0. Solution B: Tryptone, 5 g; yeast extract, 2·5 g.; $(NH_4)_2HPO_4$, 2·5 g.; L-asparagin (monohydrate), 1·0 g.; Na_2HPO_4, 0·05 g., K_2HPO_4, 0·05 g. The asparagin was dissolved separately and added to the other components dissolved in *c.* 1·5 l. of water. After the pH value had been adjusted to 6·5–7·0 the volume was made up to 2 l. Solution A, in 25 ml. amounts and solution B, in 100 ml. amounts, were autoclaved separately and mixed when cool.

Pectic substrates. For pectin methyl esterase test: Brown Ribbon pectin, 0·5 g.; phenol, 0·2 g.; NaCl, 0·58 g.; bromthymol blue solution (British Drug Houses Ltd., Poole, Dorset) 2·5 ml.; water, to 100 ml. Before use the colour of the indicator was adjusted to green by addition of N-NaOH. For viscosimetric tests: pectic acid was prepared from Brown Ribbon pectin by the method of Jermyn & Tomkins (1950). Stock 1 % (w/v) solutions of pectin and pectic acid were used to prepare the substrates summarized in Table 2.

Preparation of culture solutions for testing. Conical flasks (250 ml.) containing medium SM 1 (125 ml.) were inoculated with 1 ml. of 48 hr. cultures in medium IM 1 and incubated statically at 25°. At approximately weekly intervals samples were withdrawn and centrifuged at 1200 g for 15 min.

Tests on culture supernatant fluids

pH values were measured by glass electrode.

Ethanol precipitation. Absolute ethanol (2 ml.) was added to 1 ml. of culture supernatant fluid, shaken and centrifuged; the sediment was compared with that from an uninoculated control and graded arbitrarily.

Pectin methyl esterase. A sample (0·6 ml.) of centrifuged culture was pipetted into each of two test tubes. One was immersed in a boiling-water bath for 10 min. to inactivate the enzyme. Substrate (7·4 ml.) was added to each tube followed by dilute NaOH until the indicator was blue. The tubes were incubated for at least 24 hr. at 30°. The production of an acid reaction in the unheated tube was taken to indicate the presence of PME. The heated tube served as a control on non-enzymic acid production and as a colour standard.

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* 'Brown Ribbon' pectin is a pharmaceutical grade pectin with 12·5 % methoxyl (on weight of pectin) obtained from Union Crystalex Gelatine Ltd., Garrett Street, Golden Lane, London, E.C. 1.

Pectin glycosidase. Various substrates (Table 2) in 7·4 ml. amounts were added to 0·6 ml. of culture supernatant and mixed. After mixing the tubes were incubated in a 30° water bath for 24 hr. Viscosities were then measured by a simple pipette, similar to the Baker 'Jelmeter' (see Kertesz, 1951, p. 482), with a delivery tube of 1·6–1·7 mm. internal diameter. In any set of readings a separate pipette was used for each substrate; after a measurement it was rinsed with boiling distilled water, drained and placed on an air line while readings were taken on the other five substrates, by which time it was sufficiently cool and dry to use again. Generally loss of viscosity was obvious, being practically complete with the more active cultures. Pectin glycosidase activity was only considered to be present when the decrease of viscosity was greater than 10 % of the maximum possible.

RESULTS

Table 3. *Species showing neither pectin glycosidase nor pectin methyl esterase activity*

(*a*) Plant pathogenic species

Agrobacterium radiobacter	(1/1)	*P. mors-prunorum*	(4/4)*
A. rhizogenes	(1/1)	*P. prunicola*	(1/1)
A. tumefaciens	(2/2)	*P. syringae*	(4/5)
Corynebacterium fascians	(2/2)	*P. tabaci*	(1/1)
C. flaccumfaciens	(1/1)	*Xanthomonas begoniae*	(2/2)
C. michiganense	(2/2)	*X. hyacinthi*	(3/3)
Erwinia tracheiphila	(1/1)	*X. juglandis*	(3/3)
Pseudomonas coronafaciens	(1/1)	*X. malvacearum*	(5/5)
P. delphinii	(1/1)	*X. phaseoli*	(1/1)
P. marginalis	(2/3)	*X. pruni*	(2/2)
P. medicaginis	(1/1)	*X. vasculorum*	(3/4)
P. medicaginis var. *phaseolicola*	(1/3)		

(*b*) Non-pathogenic species

Serratia marcescens	(1/1)	*Rhizobium* sp. (? meliloti)	(4/4)
Flavobacterium aquatile	(1/1)	*Proteus vulgaris*	(3/3)
F. devorans	(1/1)	*Pseudomonas aeruginosa*	(1/1)
Klebsiella pneumoniae	(1/1)	*Pseudomonas* spp. from plant material	(3/5)
Coli-aerogenes bacteria	(26/32) (see Table 6)		

* 3 of the strains of *P. mors-prunorum* gave positive results in single tests.

The results are summarized in Tables 3–6. The figures in parentheses (*a*/*b*) refer to (*a*) the number of strains showing the property listed, (*b*) total number of strains of that species tested.

Table 4. *Species showing pectin glycosidase activity but no pectin methyl esterase*

(*a*) Plant pathogenic species

Erwinia carotovora	(1/5)	*P. medicaginis* var. *phaseolicola*	(2/3)
E. rhapontici	(1/1)	*P. syringae*	(1/5)
Pseudomonas marginalis	(1/3)	*Xanthomonas papavericola*	(1/1)

(*b*) Non-pathogenic species

Bacillus macerans	(2/2)	*Flavobacterium* sp. from soil	(1/1)
B. polymyxa	(2/2)	*Pseudomonas* spp. from plant material	(2/5)
B. subtilis	(2/2)		
Coli-aerogenes bacteria	(6/32) (see Table 6)		

Table 5. *Species showing both pectin glycosidase and pectin methyl esterase activity*

(*a*) Plant pathogenic species

Erwinia aroideae	(2/2)	*E. phytophthora*	(1/1)
E. atroseptica	(4/4)	*Xanthomonas campestris*	(3/3)
E. carotovora	(4/5)	*X. vasculorum*	(1/4)

(*b*) Non-pathogenic species
None

Table 6. *Production of pectin glycosidase by various types of coli-aerogenes bacteria*

	No of strains		Source of strains	
Type	Tested	P.G. positive	Positive	Negative
E. coli I and II	6	0	—	M, 3; U, 3
Cit. freundii I and II	11	1	W, 1	M, 3; S, 3; W, 2; U, 2
K. aerogenes I and II	9	4	S, 3; W, 1	M, 1; S, 1; W, 2; U, 1
K. cloacae	4	1	W, 1;	M, 1; S, 1; W, 1
Irregular	1	0	—	U, 1
Paracolon	1	0	—	U, 1

* Sources; M, milk; S, soil; W, water; U, unknown.

DISCUSSION

The occurrence of pectin glycosidase in the bacterial species studied in this investigation is more or less in accord with previous reports. More striking is the restricted distribution of pectin methyl esterase.

Turning first to pectin glycosidase and the various genera in which it was found, it should be noted that the pectolytic activity was of the γ-PG type; i.e. destruction of viscosity was more rapid at pH 7·5 than at pH 5·5 or 3·5 and both pectin and pectic acid were attacked. In a few instances the activities at pH 5·5 were sufficiently high to suggest that α-PG might also be present but this always accompanied very high activity at pH 7·5. The present evidence is insufficient to state definitely whether α-PG was or was not present in these cases. It is worth noting that Kraght & Starr (1953) found that an enzyme from *Erwinia carotovora* had an optimum pH of about 5·8 for the liberation of reducing groups from pectic acid, whilst Ozawa (1947) found an optimum of 5·5–6·0 for the 'protopectinase' of *E. aroideae.* Most of the cultures in which the presence of α-PG was suspected in this investigation were of the soft-rot erwinias.

Bacillus *species.* All three species tested, and found to produce PG have been reported to be active against pectic materials (Allen, 1944; Jacobelli, 1953; McCready, Owens & Maclay, 1943; Nortje & Vaughn, 1953; Ozawa, 1947; Rudd Jones, 1946), although some of these authors used gel liquefaction techniques which did not give unequivocal evidence of enzyme production or indicate the properties of any enzyme concerned. Ozawa (1947) found a pH optimum of 8–9 for the 'protopectinase' of *Bacillus natto* (=*B. subtilis*) and Nortje & Vaughn (1953) give 8·6 as the optimum for the pickle-softening enzyme of *B. subtilis.*

Pseudomonas *species.* The chief characteristic of pectic enzyme production in Pseudomonas seems to be its variability: the three positive species *P. marginalis, P. phaseolicola, P. syringae* each included inactive strains. The results of Oxford (1944) also suggest variation between strains of *Pseudomonas* spp. particularly when it is noted that Mills (1949) found PME (and so, probably, PG) to be produced by *P. prunicola,* which Oxford reported as not pectolytic (2 strains) and which also gave negative results in the present investigation, as did *P. tabaci* which Oxford and Sabet & Dowson (1951) found to be pectolytic. None of the saprophytes tested by Oxford were pectolytic but two of the strains used in the present investigation produced PG. This variability may be inherent in the species tested or some strains may have lost the faculty during laboratory cultivation; before accepting pectolytic ability as a diagnostic feature in this genus it would be necessary to make certain that the former was not the case and to prevent the latter as far as possible.

Xanthomonas *species.* Although this genus does not, apparently, possess pectolytic activity as a general attribute, it includes the only species, outside the soft-rot erwinias, found to produce pectin methyl esterase, viz. all three strains of *Xanthomonas campestris* and one strain of *X. vasculorum.* These two species and *X. papavericola,* the other producer of PG, are related serologically while the remaining species tested fall into other serological groups (Elrod & Braun, 1947). According to Sabet & Dowson (1951) *X. begoniae* and *X. malvacearum* (both negative in this investigation) caused 'slight, thick liquefaction' of their pectate gel medium, but this cannot be regarded as definite evidence of the production of pectic enzymes; *X. citri* (not tested here) and *X. campestris* (PG+, PME+) caused 'moderate liquefaction and clear liquid'. Shishelova (1951) found that *X. campestris* produced 'protopectinase' *in vitro* and *in vivo*; two other species, *X. phaseoli* and *X. translucens* produced it only in infected plants.

Agrobacterium *and* Corynebacterium *species.* No evidence of pectic enzyme production was found in these two genera, although Sabet & Dowson (1951) reported 'slight to moderate liquefaction' for *Agrobacterium tumefaciens.* According to Shishelova (1951) *Corynebacterium michiganense* did not produce protopectinase *in vivo* or *in vitro*. The nature of the diseases caused by these pathogens does not suggest that pectic degradation is involved.

Coli-aerogenes bacteria. The relationship between the soft-rot erwinias and the coli-aerogenes group of bacteria has been debated for some time (Stuart, Griffin & Baker, 1938; Stuart *et al.* 1940; Elrod, 1942; Taylor, 1951). Taylor found that of 19 soft-rot bacteria akin to *Erwinia carotovora*, 17 liquefied pectate gel whilst none of some 300 other coli-aerogenes bacteria did so. So far, however, the enzymes involved in gel liquefaction have not been identified or the possibility of non-enzymic softening excluded. Some caution is therefore necessary in applying this as a diagnostic test, although the method is undoubtedly valuable for isolating or enumerating potentially pectolytic organisms (Wieringa, 1949; Elis Jones, 1956). It is noteworthy that all six PG-producing coliform organisms were isolated from soil or water and that 5 of them were of the aerogenes-cloacae type most closely related to *E. carotovora*.

Pectin methyl esterase was more restricted in its distribution than pectin glycosidase and was never found in the absence of the glycosidase. Although *Pseudomonas prunicola* showed neither PG nor PME in this investigation Mills (1949) found it to be an active producer of the esterase when grown in the presence of pectic material. Fabian & Johnson (1938) claimed to have demonstrated that members of the *Bacillus mesentericus-megaterium* group produced pectase (=PME) and protopectinase but not pectinase (=PG) but gave no experimental details of their methods or results. Kraght & Starr (1953) showed production of PME by five strains of *Erwinia carotovora*, but Wood (1955) concluded that his culture of *E. aroideae* was essentially free from PME. During the present investigation it has been observed that formation of PME is much more sensitive to variations in the cultural conditions than is PG formation. The fact that Wood used a simple medium containing no pectic material probably explains the absence of PME from his culture solutions. Whilst the study of an individual enzyme is facilitated by the use of a medium selective for its production the whole pectolytic complex must be kept in view when the disease processes are being considered. Nutrient conditions in the infection count are far from simple and pectic substances are almost certainly present in forms which will induce adaptive enzyme formation. Production of PME seems likely and its role in soft-rotting is discussed in an accompanying paper (Smith, 1958).

In summary it can be said that a number of saprophytic bacteria as well as some plant pathogenic species produce enzymes which degrade pectic substances but production of PME is confined to a small number of pathogens. These are the soft-rot *Erwinia* spp. and *Xanthomonas campestris*, in each of which it might well be a diagnostic feature, and *X. vasculorum*, where the faculty is less constant.

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Chromatographic Examination of the Products of Digestion of Pectic Materials by Culture Solutions of Plant Pathogenic and other Bacteria

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SUMMARY: Solutions containing pectic materials were digested by solutions from cultures of 25 bacteria and examined chromatographically for breakdown products. Galacturonic acid and oligo-uronides were found with soft-rot *Erwinia* spp., *Xanthomonas campestris*, *Pseudomonas marginalis*, *Bacillus polymyxa* and *Klebsiella aerogenes* (galacturonic acid only).

In an accompanying paper (Smith, 1958) the results of screening a number of cultures of bacteria for production of pectic enzymes have been described. To obtain further information on the nature of the enzymes concerned the products of digestion of pectic materials by some of the bacteria were examined by paper partition chromatography.

METHODS

Cultures were grown in SM1 medium (Smith, 1958) and sampled at various times. The supernatant fluid after centrifuging was added to substrates of pectin or pectic acid at pH 7·5 or 5·5 as for viscosity tests—in some cases the proportion of culture was increased from 7½ to 10 %—and incubated for periods of up to 3 days. In most cases loss of viscosity was practically complete in the first 24 hr. but further diminution in the ethanolic precipitate occurred on longer incubation.

Samples for chromatography were concentrated and purified because tailing of the crude digests made identification of spots on the chromatograms uncertain. The following simple procedure purified the solutions by removing higher uronides and some unidentified material which practically coincided with galacturonic acid on the chromatograms. Absolute ethanol (5 ml.) was mixed with 5 ml. of digest and the precipitated colloidal material centrifuged off. The calcium salts of the low oligo-uronides were then precipitated by adding a drop of 1 % $CaCl_2$ solution and 10 ml. of absolute ethanol. The precipitate was centrifuged down, drained and suspended in 1 ml. of distilled water. Solution was completed by adding a pinch of Amberlite I.R. 120 cation exchange resin in the hydrogen form; 0·1 ml. of this solution was spotted on the chromatogram. Marker spots on the chromatograms were (*a*) 5 μl. of a 0·5 % (w/v) solution of galacturonic acid; (*b*) 50 μl. of a solution containing galacturonic acid and di-galacturonic acid, obtained by digestion of pectic acid with fungal pectin glucosidase (PG) and purified by ethanol precipitation.

Controls of substrate without culture filtrate were run with the test solutions, but controls of substrate + inactivated culture supernatant were omitted to obtain the maximum number of test spots on each paper. This was considered to be justified as preliminary tests had indicated that the concentrations of galacturonic acid and oligo-uronides in the culture fluids were low. Kraght & Starr (1952) have shown that galacturonic acid can be metabolized by *Erwinia carotovora* and so it would not be expected to accumulate in the culture medium. The conclusion that a particular culture could degrade pectic substances to low uronides would be the same whether these uronides were derived from the culture or from subsequent digestion of a substrate solution by culture supernatant.

The solvent used was a modification of the ethyl acetate + acetic acid + water system (Jermyn & Isherwood, 1949; Ayres *et al.* 1952; Demain & Phaff, 1954; McCready & McComb, 1933). With a mixture of equal volumes of the three components galacturonic acid moved approximately 30 cm. when developed (descending) overnight on Whatman no. 1 paper. The di-, tri- and tetra-uronides were spaced behind the galacturonic acid so that there was a constant ratio of 0·76–0·84 between the distances moved by successive members of the series. After development the papers were dried, sprayed with benzidine + trichloroacetic acid reagent (Roelofsen, 1953) and heated at 90°–105° for 10 min. Under these conditions 5 μg. galacturonic acid gave a definite brown spot.

RESULTS

The results are summarized in Table 1.

To confirm the presence of galacturonic acid and obtain some idea of the concentration the Dische (1947) reaction was applied to some of the solutions used for chromatography. By comparison with standard solutions of galacturonic acid it was estimated that some 10 % of the original pectic material was present as monomer or dimer in digests by soft-rot *Erwinia* spp., with individual cultures ranging from 2 % to 20 %.

DISCUSSION

These results show that, like fungi and yeasts, some bacteria produce enzymes capable of breaking down pectic substances to galacturonic acid and the low oligo-uronides. This faculty is distributed similarly to the ability to produce pectin methyl esterase (PME) but there are some exceptions—galacturonic acid is liberated in the absence of detectable PME by one strain of *Erwinia carotovora* and by *Bacillus polymyxa*, *Klebsiella aerogenes*, *Pseudomonas marginalis*, *P. medicaginis*, whilst the strain of *Xanthomonas vasculorum* which produced PME did not liberate low uronides.

Pectic enzymes produced by the soft-rot Erwinia *spp.* Kraght & Starr (1953) have shown that *Erwinia carotovora* produces PME and PG activity which, by measurement of increase in reducing power, has a pH optimum of 5·8: the viscosity and ethanol precipitability of pectin solutions were also

Table 1. *Production of galacturonic acid and low uronides from pectic substances by various bacteria*

Organisms	No. of strains	Production of (1)		Galacturonides liberated	
		Glycosidase	Esterase	Mono	Higher (2)
Erwinia carotovora, (3) etc.	8	+	+	+	+
E. carotovora	1	+	−	+	+
Xanthomonas campestris	1	+	+	+	+
X. vasculorum	1	+	+	−	−
E. raphontici	1	+	−	−	−
Klebsiella aerogenes	2	+	−	+	−
Pseudomonas marginalis	1	+	−	+	+
P. medicaginis	1	+	−	+	+
P. syringae	1	+	−	−	−
Non-pathogenic *Pseudomonas* sp.	1	+	−	−	−
X. papavericola	1	+	−	−	−
Flavobacterium sp.	1	+	−	−	−
Bacillus polymyxa	1	+	−	+	+
B. subtilis	1	+	−	−	−
E. tracheiphila	1	−	−	−	−
Escherichia coli	1	−	−	−	−
Corynebacterium flaccumfaciens	1	−	−	−	−

(1) (Smith, 1958).
(2) May include di-, tri-, tetra-, penta-galacturonic acids.
(3) Includes *E. aroideae*, *E. atroseptica*, *E. phytophthora*.

destroyed. Wood (1951, 1955*a*) has described preparations of *E. aroideae* which destroyed the viscosity of pectin and pectic acid most rapidly at pH values above 7. They also liberated reducing groups but no galacturonic acid could be detected in the products and PME was absent or negligible in amount. In the present investigations (Smith, 1958) no qualitative differences have been found in the pectic enzymes produced by the various soft-rot bacteria—*E. aroideae*, *E. atroseptica*, *E. carotovora*, *E. phytophthora*; all produce PME, destroy the viscosity and ethanol precipitability of pectin and pectic acid solutions and liberate galacturonic acid from pectic substances. Destruction of viscosity is more rapid at pH 7·5 than 5·5, but, in some cases, activity at the lower pH is sufficient to suggest the presence of α-PG. Whilst it thus appears that the soft-rot *Erwinia* spp. produce two pectin glycosidases the possibility remains that the same enzyme might have different pH optima depending upon the method of assay. The results of Phaff & Demain (1956) with a purified yeast polygalacturonase (YPG) are relevant in this connexion. YPG hydrolyses pectic acid with an optimum pH of 4·4 at which value tetra-galacturonic acid and tri-galacturonic acid are attacked very slowly. At pH 3·5 the breakdown of these uronides under the influence of YPG is accelerated many fold. As a result lowering the pH from 4·5 to 3·5 will decrease the rate at which the viscosity of a pectic acid solution is destroyed and increase the rate of liberation of reducing substances.

Role of pectin methyl esterase in soft-rot diseases. The function of PME in rotting is not so obvious as that of PG particularly when the pathogen produces glycosidases capable of attacking pectic substances of both high- and low-methoxyl content. Nevertheless, it is possible that PME, directly and indirectly, plays an important part in the rotting process and might even, as its restricted distribution suggests, determine the difference between pathogenicity and non-pathogenicity.

Indirect effect of PME. Whether or not an infection develops into a lesion is determined by the relative speeds of the pathogenic processes of the parasite and the defensive mechanisms of the host. Anything favouring the former will increase the probability that a rot will become established. Lapwood (1957) has shown that one difference between soft-rot *Erwinia* spp. and some saprophytic PG-producing bacteria is the greater growth rate of the former in potato media. If, in addition, the action of the PG of the pathogens was accelerated this difference would be enhanced. Wood (1955*a*) suggested that high- and low-methoxyl pectins are degraded at similar rates by PG from *E. aroideae* but the experimental figures he gave indicate important differences. Hydrolysis, relative to the theoretical maximum possible increase in reducing power, was: at 5 min., pectin, 1·7 %; sodium pectate, 10·0 %; at 24 hr., pectin, 19·1 %; sodium pectate, 37·4 %; after this little further increase occurred. These differences are hardly negligible but, in fact, indicate that demethylation increases the susceptibility of the substrate to enzymic hydrolysis. The final extent of degradation is nearly doubled, and, more significant as far as rotting is concerned, initial breakdown is more rapid.

Direct effect of pectin methyl esterase. Whilst direct evidence is lacking the probable result of de-esterification of the plant pectic colloids would be to reduce their water-holding capacity (Kertesz, 1951, p. 184; Deuel & Solms, 1954) resulting in a condition similar to water-soaking. This has been shown (Fernando & Stevenson, 1952; Gregg, 1952; Lapwood, 1957) to render vegetable tissue susceptible to attack by bacteria which do not produce rots in normal tissue, e.g. *Bacillus subtilis*, *Pseudomonas syringae*, *Flavobacterium* sp. —organisms which differ from the soft-rot *Erwinia* spp. in not producing PME (Smith, 1958).

Role of pectin glycosidase in soft-rot diseases. Destruction of the colloidal pectic material of plant tissue plays an obvious part in soft-rotting and this aspect has been discussed many times (see, for example, Wood, 1955*b*). Galacturonic acid, which is probably liberated, can be metabolized by *E. carotovora* (Kraght & Starr, 1952).

Extent of glycosidase action. The figure given by Wood (1955*a*) for the ultimate extent of hydrolysis of pectic acid, 37 %, is interesting in indicating that the final products have an average size of 2·7 galacturonic acid units. If no galacturonic acid is present these products must consist of a mixture equivalent to 30 % di-galacturonic acid and 70 % tri-galacturonic acid (by number). If higher polymers are present the proportion of di-galacturonic acid molecules must be increased considerably. This suggests that the extent of hydrolysis is limited not by some hypothetical unit in the pectic molecule but by a restriction on the capabilities of the enzyme, e.g. that, like β-amylase, it cannot attack a terminal glycoside bond. In view of these considerations Wood's choice of the term 'depolymerase' is unfortunate, despite his reservation that it 'in no way attempts to specify the way in which the enzymes act': more particularly so since the enzyme to which Kertesz (1949) applied the term did not attack high-methoxyl pectin but required the prior action of PME and was inhibited by calcium (McColloch & Kertesz, 1948). Schubert's term 'pectinglycosidase' seems to be more appropriate, at least until the mode of action of the various enzymes is more exactly understood.

Organisms other than soft-rot Erwiniae. Little is known about the pectic conversions of the other bacteria examined, and it is difficult to assess the significance of the chromatographic findings at present. In the case of the two strains of *Klebsiella aerogenes* only small amounts of galacturonic acid and no higher uronides were detected. The rate at which they reduced the viscosity of pectic solutions was slower than that of the soft-rot erwinias so that it seems possible that their enzymes operate by stepwise removal of terminal units rather than by random fission of the molecule. They would thus be similar to the galacturogenic polygalacturonase of *Aspergillus niger* (Saito, 1955).

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Intracerebral Infection of Mice with *Haemophilus pertussis* and Passive Protection by Hyperimmune Rabbit Sera

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SUMMARY: The intracerebral invasion in mice by two *Haemophilus* (*Bordetella*) *pertussis* strains, a virulent strain and the strain 18–323 which is very virulent on intracerebral injection, was studied. The LD50 of these strains differed by about a thousand times and the average slopes of the regression lines from which these were calculated differed significantly, ($P=0{\cdot}04$ and $P=0{\cdot}01$). It was found that the number of organisms/mouse brain in infected animals reached a maximum count which was slightly higher for the more virulent strain. The day on which the maximum count was reached depended both on the dose of organisms and the strain used, but occurred always before the seventh day after injection. Further, a slightly lower maximum count was reached as the dose of organisms was decreased. The passive protection by rabbit sera, described as antibacterial and antitoxic sera, against both these strains of pertussis organisms, was studied. It was shown that the protection of neither type of serum was due only to the presence of agglutinin antibodies.

The intracerebral infection of mice (Norton & Dingle, 1935) in preference to the intranasal instillation (Burnet & Timmins, 1937) was used by Ospeck & Roberts in 1944 with virulent *Haemophilus* (*Bordetella*) *pertussis* organisms because of its precision in administration. By this method they challenged the passive protection conferred by hyperimmune rabbit sera in mice. Kendrick, Eldering, Dixon & Misner (1947) described a strain of *Haemophilus pertussis*, 18–323, which was very virulent to mice by the intracerebral route. Small doses of this strain were used to challenge mice actively immunized with different doses of vaccines. Further work which was published in 1949 by Kendrick, Updyke & Eldering led to the current method for the standardization of pertussis vaccines (M.R.C. Report 1956). Invasion of mouse brain by the organism was not extensively studied by any of these workers; it has now been investigated by using a virulent pertussis strain and the virulent strain 18–323.

METHODS

Strains of Haemophilus pertussis *used.* Two strains of pertussis organisms were used for the study of intracerebral invasion in mice and as challenge organisms against passive immunity in mice. The first strain, a smooth virulent freshly isolated one (no. 1011) which had been kept in the dried state, was obtained from the National Collection of Type Cultures; the second was the smooth virulent strain described by Kendrick *et al.* (1947) and was obtained from two sources and numbered, respectively, 1016 and 1017.

Experimental animals. Male Swiss breed mice weighing between 17 and 20 g. were used for all experiments. Albino guinea pigs weighing not more than 350 g. were used for the intracutaneous testing of pertussis toxins and antitoxins. Rabbits weighing between 2 and 4 kg. were used for the production of hyperimmune antisera.

Antigens used to prepare hyperimmune sera. Vaccines containing approximately 10,000 killed smooth pertussis organisms/ml. were used as antigens in the production of antibacterial sera.

Toxic filtrates from the growth of smooth pertussis organisms in a meat infusion made with a plain papain digest of horse muscle (Linggood & Fenton, 1947) containing 0·25 % starch, similar toxic filtrates with the addition of 0·4 % (w/v) potash alum, and toxic extracts of organisms similarly grown, as prepared by Evans & Maitland (1937), were used in the production of antitoxic sera.

Preparation of hyperimmune sera

Antibacterial sera. Group 1: up to ten graded doses of vaccine from 0·1 to 1·0 ml./kg. rabbit were given to rabbits by the intramuscular route at 2-day intervals, with a rest of 1 month after the first two doses. Group 2: at least two courses of ten graded doses as above were given to rabbits, either intramuscularly or intravenously. The rabbits from both groups were bled on the fourth day or on the fourth and seventh days after injections. The blood serum obtained was clarified by rapid centrifugation or by sterilization through seitz EK filter pads.

Antitoxic sera. Rabbits were given by the intramuscular route graded doses of toxic filtrates, toxic filtrates with alum or toxic extracts, until the antitoxic potency of their blood serum ceased to increase. It was shown by Roberts & Ospeck (1944) that antitoxic sera prepared against toxic filtrates from pertussis organisms neutralized the toxicity of toxic extracts from these organisms. Before use in protection experiments the blood sera from the fully immunized rabbits were absorbed with suspensions of smooth living pertussis organisms containing at least 20,000 million organisms/ml. After absorption the sera were heated at 56° for 2 hr. held at 4° for at least 24 hr., and finally clarified by centrifugation or filtration through Seitz pads. When the agglutinin titre had not been decreased to 100 or less by one absorption the process was repeated.

Standardization of materials

Preparation of suspensions and estimation of their total number of organisms. All cultures were kept in the dried state and were recovered by incubation at 37° for 48 hr. and a further subculture for 24 hr., all on 50 % (v/v) sheep blood Bordet-Gengou medium. Suspensions made from organisms in Casamino acid broth (Cohen & Wheeler, 1946) were standardized by visual comparison with a modification of the scale of opacities suggested by Brown & Kirwan (1915). The suspension equivalent in opacity to opacity 4 on this scale was designated the standard suspension. It was found to contain approximately 8×10^9 organisms/ml. when counted in a Thoma bacterial counting cell.

Determination of the LD50 of each strain. The stability of each strain was checked at intervals throughout the work by the determination of its LD50 when injected in 0·05 ml. by the intracerebral route. Groups of 10 mice for each test dose of culture were observed during 21 days after injection. The results were calculated by the method of Reed & Muench (1938).

Plate counts of pertussis organisms. Comparisons of the approximate number of live organisms in suspensions were made by the method of Miles & Misra (1938). Bordet-Gengou sheep-blood medium containing 1 unit penicillin/ml. in plates of at least half a centimetre in thickness was used for growth of the organisms. Tests with organisms from a number of dryings of both strains showed that in comparisons made at different times the maximum number of organisms counted in one ml. of a standard suspension was $4{\cdot}9 \times 10^9$ and the minimum $1{\cdot}2 \times 10^9$. From the total estimated number of 8×10^9 organisms/ml. of standard suspension it may be deduced that between 15 and 60 % of these organisms were viable.

Potency of antibacterial sera. The agglutinin titres were measured by using suspensions of live pertussis organisms and incubating the mixtures of serum and culture at 37° overnight. Each serum when tested for the presence of antitoxin was found to contain less than one unit/ml.

Potency of antitoxic sera. The antitoxic titres of these sera were estimated by comparison with a serum standardized according to the unit of antitoxin defined by Roberts & Ospeck (1944). In neutralization experiments any excess toxin present in a series of toxin-antitoxin mixtures could be indicated by death in mice, or by necrosis in rabbit skin (Roberts & Ospeck, 1944) or by necrosis in guinea-pig skin. The latter method was chosen in this work. Guinea pigs with very thin skins were rejected as poor reactors. A scale in which the volume of each dose differed from the next by 20 % was used for the serum dosage. Sera standardized in this way were known to contain antibodies other than antitoxin as they were prepared against impure antigens.

RESULTS

Comparisons of the relationship between the intracerebral dose in mice of virulent pertussis strain 1011 *or of the virulent pertussis strain* 1017 *and the mortality of the mice injected*

Similar suspensions of each of the above strains of pertussis organisms were made from organisms recovered from the dried state at the same time and subcultured on the same batch of Bordet-Gengou medium. Ten or 20 mice from one population of animals were injected intracerebrally for each of at least five doses of suspension. The animals were kept in the same part of the mouse room and observed for 21 days. Four comparisons were made in this way and the results are given in Table 1. The slopes of the regression lines (Bliss, 1935) were determined by the method of Fisher & Yates (1949) and the LD50 of each strain were calculated from the regression lines. The LD50 found in this way agree with the values around 400 organisms for strains 1016 and 1017 and 200×10^3 organisms for strain 1011, determined at intervals during the work by the method of Reed & Muench (1938). In the hope of finding another strain characteristic, the differences in the slopes of the regression lines for the two strains were compared for their significance with their standard errors by means of *t*-tests. The results (giving respectively a percentage possibility of similarity of slope of 58, 50, 55 and 50) show that there was no significant difference between them when each experiment was analysed separately. However, in a block analysis, using the

Table 1. *Variance of mortality in mice from a series of doses of pertussis strains* 1011 *and* 1017 *injected intracerebrally*

Doses expressed as estimated total number of organisms. The slopes of the regression lines, and their standard errors, also the LD 50, were determined for each experiment.

Expt. ...		1	2	3	4
		Dead/10*		Dead/20	
Dose of organisms					
Strain 1011	2×10^2	—	—	0	2
	10^3	2	1	4	0
	10^4	2	3	5	6
	10^5	5	4	6	17
	10^6	10	6	14	20
	10^7	9	9	20	20
LD 50		96×10^3	400×10^3	320×10^3	32×10^3
Slopes of lines		0·668 ± 0·416	0·388 ± 0·278	0·628 ± 0·329	0·982 ± 0·542
		Dead/10		Dead/20	
Strain 1017	4×1	—	—	1	0
	10	1	2	1	8
	10^2	8	9	6	16
	10^3	9	9	18	20
	10^4	9	9	19	19
	10^5	8	10	20	20
LD 50		200	200	504	104
Slopes of line		1·251 ± 0·998	0·846 ± 0·576	1·026 ± 0·539	1·446 ± 0·352

* Figures are dead mice/group of 10 or/group of 20.

mean slopes of the lines, it was found that a *t*-test comparing the difference between the mean slopes of strains 1011 and 1017 with the standard error of this difference gave a significant result $P = 0{\cdot}04$, indicating that in only 4 % of trials would the mean slopes be alike. Finally, when the hypothesis that there is no real difference between the slopes of the corresponding lines is considered, the mean difference between the slopes of the lines found for the two cultures when examined on the same day in the same population of mice should be zero. The *t*-test comparing the mean differences with the standard error of the mean difference gave the result $P = c.\ 0{\cdot}01$ indicating that only in 1 % of trials the results could be zero. This confirmed that the average slopes differed significantly, and established that the slopes of the regression lines determined as above are strain characteristics. As a matter of interest similar comparisons were made on two occasions by instilling the two strains intranasally. An average LD 50 of 600×10^4 organisms for strain 1017 and of 200×10^3 organisms for strain 1011 indicated that by this route strain 1011 was slightly more lethal than strain 1017. The slope of the regression lines determined by the method of Fisher & Yates (1949) were such that in 86 to > 90 % of trials these would be the same. Further analysis was unnecessary.

The intracerebral invasion in mice with organisms from strains 1011 *and* 1017

A series of experiments with each strain was arranged so that similar suspensions of organisms were injected intracerebrally into mice from the same population on one day. Thus on one day two groups of mice would receive respectively 0·05 ml. standard opacity suspension of strains 1017 and 1011, while on a second day two groups would receive a suspension of 1/10 standard opacity of these strains. In all, suspensions decreasing from standard opacity to standard opacity $\times 10^{-9}$ were tested. Groups of 30 to 75 mice were injected with each dilution of culture used to provide sufficient animals for a selection of four live animals from each group at intervals after injection. The day on which, owing to the deaths of animals, the selection ceased to be a random one, was noted for each dose of culture. All mice damaged by injection were discarded on the first day. Mice used for counts of pertussis organisms in the brain were killed with coal gas and their brains removed aseptically and disintegrated by the method used by Proom (1947) for the lungs of mice. Up to five dilutions of the original brain suspensions were used for plate counts by the method of Miles & Misra (1938). Each plate was used for counts on two mice and each count was made in duplicate so that four plates were used for four mice. Differences in the numbers of viable organisms injected in each dose are reflected in the 1 hr. counts of the strains. These were due to the different challenge suspensions and the percentage of viable organisms in these different suspensions. The growth of the organisms from the two strains is compared in Figs. 1 and 2. The differences in the rates of growth and maximum numbers may be due to a fundamental difference in the metabolic requirements of these organisms. The falling off of the number of organisms after the third or fourth day with strain 1011 and the decrease in the maximum count reached after this time with strain 1017 may be due to phagocytosis. The shape of the curves will be determined by the balance between growth and removal of the organisms, and is characteristic for each strain.

The protection by antibacterial and absorbed antitoxic sera against lethal intracerebral doses of pertussis strains 1011 *and* 1017

Intraperitoneal graded doses of sera were given 24 hr. before intracerebral challenge with culture. In each series of protection experiments against any particular dose of culture, the numbers of mice/dose of serum were equal and varied from 5 to 30 mice/serum dose according to the volume of serum available. The protective dose of serum giving 50 % end-points (PD 50) were calculated for each challenge dose of culture by the method of Reed & Muench. Results are given for three antibacterial sera and three absorbed antitoxic sera

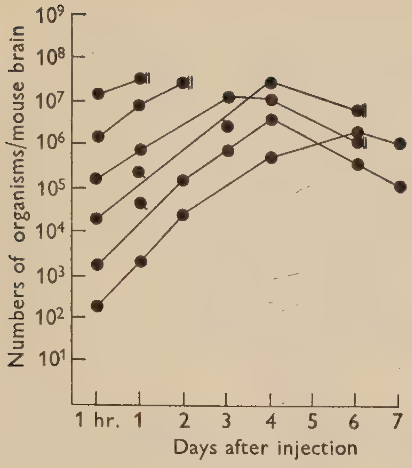

Fig. 1

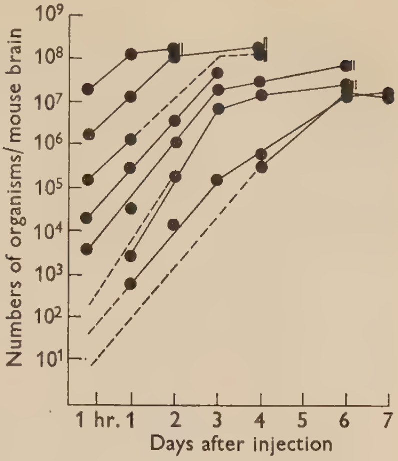

Fig. 2

Fig. 1. Average counts of pertussis organisms recovered from mouse brain after intracerebral doses of strain 1011. || Indicates first day on which deaths of mice occurred.

Fig. 2. Average counts of pertussis organisms recovered from mouse brain after intracerebral doses of strain 1017. || Indicates first day on which deaths of mice occurred. ----, Direction of graphs deduced by comparison with others of the series.

Table 2. *Fifty % end-points of protection with antibacterial and absorbed antitoxic sera against intracerebral challenge doses of pertussis strains* 1017 *and* 1011.

		Strain 1017					Strain 1011	
		Challenge × LD 50						
Antibacterial serum	No. mice/ serum dose	1×10^2	1×10^3	1×10^4	1×10^5	1×10^6	1×10^2	1×10^3
		Reciprocals of PD 50*						
No. 1	10	.	13	.	<5	.	15	<5
No. 2	10	23	24	.	13	< 5	>40	8
No. 3	30	23	13	<10	.	.	.	.
Antitoxic serum								
No. 6	10	.	10	*c.* 13	10	*c.* 5	>13	5
No. 7	8	8	.	.	8	<3	8	<3
No. 8	30	6	9	.	.	.	.	.

Agglutinin titres of sera 1–3 = 1000–2000. Antitoxic titres of sera 6–8 = *c.* 500 units. Agglutinin titres of sera 6–8 = < 100. * Each mouse received 0·2 ml. serum intraperitoneally.

in Table 2 as the reciprocals of these doses. The antitoxic sera were diluted between 2 and 3 times during absorption accounting for the maximum PD 50 of *c.* 10 for these sera in contrast to maximum PD 50 of *c.* 20 for the antibacterial sera. The approximately constant PD 50 against increasing doses of strain 1017, which are particularly evident for sera 6 and 7, are difficult to understand. An experimental investigation by means of brain culture counts would probably elucidate this problem.

The partial protection by natural and absorbed antibacterial and antitoxic sera in mice against intracerebral challenge with pertussis strains 1017 *and* 1011

Agglutinins were absorbed from both antibacterial and antitoxic sera, and protective tests with the natural and absorbed sera gave the results in Table 3. Serum 2 was tested against one other dose of strain 1017 with similar results, and serum 7 against one other dose of both strains 1011 and 1017. From these results it seems unlikely that the agglutinin antibody contributes greatly to the protective properties of either antibacterial or antitoxic sera. This work was considered necessary since the antibacterial sera used in all the previous experiments were natural sera, while agglutinins were absorbed from the antitoxic sera.

Table 3. *Fifty % end-points of protection with natural and absorbed antibacterial and antitoxic sera against intracerebral challenge with pertussis strains* 1011 *and* 1017, *compared with the agglutinin titres of these sera*

	Natural	Absorbed
Antibacterial serum 2		
*Reciprocals of PD 50	8·5 7·0	7·5 5·5
Agglutinin titres	1000	100
Antitoxic serum 7		
*Reciprocals of PD 50	12 10·5	8 8
Agglutinin titres	500	100

* PD 50 calculated from dead/groups of 6, 10, 8 and 8 mice respectively. Each mouse received 0·2 ml. serum intraperitoneally.

The comparison of the partial protection in mice against a dose of virulent pertussis organisms injected intracerebrally and instilled intranasally

The results given in Table 4 show that both antibacterial and antitoxic sera protect similarly against a virulent dose of pertussis organisms whether administered by the intracerebral or intranasal route. The same protective antibodies appear to be the active factors, however the challenge culture is administered.

Table 4. *Fifty % end-points of protection with an antibacterial serum and an absorbed antitoxic serum against intracerebral and intranasal challenge*

$10^2 \times$ LD 50 of *Haemophilus pertussis* strain 1011 administered 24 hr. after serum.

	Challenge	
	Intracerebral	Intranasal
	*Reciprocals of PD 50	
Antibacterial serum 10		
Antitoxic titre: none		
Agglutinin titre: *c.* 1000	23	18·5
Antitoxic serum 11		
Antitoxic titre: 700 units/ml.		
Agglutinin titre: 20–50	8·7	9·3

* PD 50 calculated from dead/groups of 30 and 20 mice respectively. Each mouse received 0·2 ml. serum intraperitoneally.

DISCUSSION

It is of interest that the highest average counts of pertussis organisms found in mouse brains after lethal intracerebral doses were approximately constant, in view of the results obtained by workers on anthrax. Keppie, Smith & Stanley (1953) found that the total yield of bacilli collected from successive batches of 100 guinea-pigs dying of anthrax was remarkably constant. Keppie Smith & Harris-Smith (1953) also found that similar total counts of anthrax organisms were found in six guinea-pigs dying of anthrax. In the present work the maximum count of pertussis organisms obtained appeared to be somewhat dependent on the strain used and slightly lower with the less virulent strain. In an investigation of the counts of pertussis organisms in the lungs of mice after instillation of a lethal and non-lethal dose of a particular virulent strain Proom (1947) found maximum counts between 7 and 14 days. Cooper (1952) with a non-lethal dose of an unspecified virulent pertussis strain found maximum count in the lung between 7 and 8 days. With the virulent pertussis strains used in the present work and under the particular experimental conditions described, it has been found that the day on which maximum count of organisms in the brain was obtained varied with the dose of organisms and with the strain used. The larger the dose of organisms the earlier the maximum count which always occurred before the seventh day.

The nature of the antigen(s) which produces protective antibodies is not known. The haemagglutinogen isolated by Masry (1952) and the capsular material described by Evans & Adams (1952) did not produce protective antisera. Further, the results on the protection in mice by an antibacterial and an antitoxic serum after absorption of their agglutinins indicate that neither serum was only, if at all, dependent for its protective properties on the presence of agglutinin antibodies. It would be unwise to suggest how the protective antigen described by Pillemer (1950), and produced on a large scale by Pillemer, Blum & Lepow (1954) for clinical trials, protects against challenge cultures. Evans & Perkins (1954*a*, *b*, 1955) described an interference immunity to intracerebral invasion and this immunity has been confirmed as acting similarly against the intranasal invasion of pertussis organisms by Fisher (1955). From the point of view of practical protection against natural invasion by *Haemophilus pertussis* in an animal it may be immaterial which type of antigen is used to stimulate the animal, or which type of antibody is produced. Both antibacterial and antitoxic sera have been shown to protect similarly against a small number of lethal doses of strain 1011 injected intracerebrally or instilled intranasally. However, from the viewpoint of standardization of vaccines a more complete knowledge of all antigens would be advantageous.

I am grateful to Mr A. F. B. Standfast (Lister Institute, Elstree) for considerable help with the presentation of these results, and to Dr L. F. Hewitt for interest in the work.

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The Heat Inactivation of Vaccinia Virus

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SUMMARY: Heat inactivation curves of vaccinia virus between 50° and 60° indicate that the virus is heterogeneous in its heat sensitivity. The proportion of heat-resistant particles varies inversely with the temperature of exposure. The inactivation of heat-sensitive virus is temperature dependent and seems to be a first-order reaction, while the heat-resistant fraction is inactivated at a constant slow rate unrelated to temperature over the range 50°–60°.

The work reported here is part of a larger investigation of virus inactivation by physical agents including ultraviolet and ionizing radiations, under conditions that do not destroy antigenicity (McClean, 1945; Collier, McClean & Vallet, 1956). The heat inactivation of vaccinia virus was determined as a preliminary to testing the protective antigenicity of heat inactivated vaccines.

METHODS

Virus. Most of the experiments were done with the Lister Institute strain of vaccinia virus adapted to the chick embryo; two experiments were done with the WR mouse neuro-adapted strain of virus. Infected chorioallantoic membranes were extracted in dilute McIlvaine buffer by grinding with sterile powdered neutral glass. Extracts were centrifuged at 1000 *g* for 15 min. to clear them of debris, titrated and stored at 4°. For inactivation, extracts were diluted to a titre between about 10^7 and 10^8 infective units/ml.

Experimental procedure. One ml. samples of virus were heated in small, thin-walled test tubes in a water bath accurate to $\pm 0{\cdot}2°$. Samples were removed from the bath at intervals and immediately cooled in a melting ice bath. They were stored at 4° until infectivity titrations were made: this was generally within a few hours, but on a few occasions there was an interval of 2 or 3 days.

Infectivity titrations were made by pock counts on the chorioallantoic membranes of 12- or 13-day chick embryos. The refinements of membrane handling technique described by Westwood, Phipps & Boulter (1957) were used. In titrations of WR strain 3-day instead of the usual 2-day incubation was used, because after 2 days the pocks were pale and poorly developed.

Diluent. All extractions and dilutions were made in McIlvaine's phosphate + citrate buffer, pH 7·2, 0·004 M-phosphate.

In graphs of the results each point is the arithmetic mean of at least four experiments unless otherwise stated. The results are plotted as log V_0/V against time, where V_0 is the original concentration and V the concentration at any time t. This enables all the inactivation curves to be given a common origin for ease of comparison, and it is also one of the terms of the first-order reaction rate equation: $\log V_0/V = Kt/2{\cdot}3$.

RESULTS

At 60° there is an initial rapid inactivation of most of the virus, and a slower inactivation of a small residual fraction. An experiment was done to see whether this greater resistance to heat was genetically determined. From a membrane inoculated with a sample heated for 60 min. a single pock was taken and extracted in 1 ml. of buffer. The bacteriologically sterile extract was passaged undiluted on the chorioallantoic membranes of five 12-day embryos and 2 days later the virus was extracted from the infected membranes. Fig. 1 shows the course of inactivation at 60° of the original virus and of the clone derived from the single pock. In four tests the inactivation at 60° of preparations descended from 'heat-resistant' virus did not differ from that of the original virus.

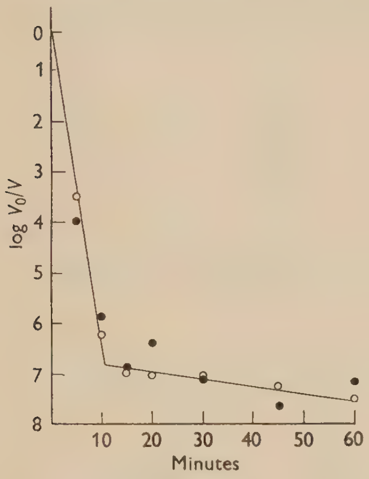

Fig. 1

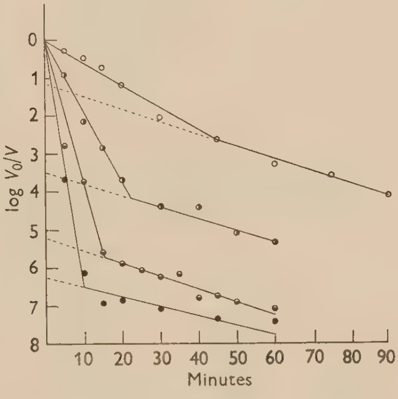

Fig. 2

Fig. 1. Inactivation of vaccinia virus at 60°. ○, 'Original virus'; 3 experiments; ●, progeny of 'heat resistant' virus: 4 experiments; V_0, initial virus concentration; V = virus concentration at time t.

Fig. 2. Inactivation curves of vaccinia virus extrapolated to theoretical initial concentration of heat-resistant fraction: ○, 50°: 6 experiments; ◑, 52·5°: 4 experiments; ◒, 55°: 6 experiments; ●, 60°: 7 experiments.

The clone derived from 'heat-resistant' virus was inactivated at 50°, 52·5° and 55°. Over the range 50°–60° inactivation appeared to proceed as a first-order reaction until more than 90 % of the infectivity had been destroyed (Fig. 2). The inactivation curves suggest two processes, and are consistent with the view that the second process not only succeeds the first but may start simultaneously with it. The velocity constants of both the fast and the slow inactivation, calculated for each temperature (Table 1), show that the rate of fast inactivation is dependent on temperature, and that of the slow inactivation of the heat resistant survivors is apparently independent of temperature—at least over the 10° range investigated. Fig. 3, a composite of several experiments at 50°, shows two inflexions, indicating two changes of inactivation rate. This phenomenon was observed in four experiments at 50° and may indeed prove to be real. In this connexion it may be noted that we have obtained similar double inflexions in the curves of inactivation of vaccinia virus by γ rays (unpublished work). This was not looked for at any other temperature.

Table 1. *Velocity constants of fast and slow inactivations of vaccinia virus showing temperature dependence of fast reaction* (K_f) *and lack of temperature dependence of slow reaction* (K_s)

Temperature (°C.)	K_f (min^{-1})	K_s (min^{-1})
50	0·14	0·07
52·5	0·42	0·073
55	0·87	0·074
60	1·48	0·056

Table 2. *Activation energies* (E) *of heat-sensitive fraction showing decrease of E as the temperature of exposure increases*

Temperature range (°C.)	E (cal./mole/°C.)
50–52·5	91,800
52·5–55	61,600
55–60	23,000
50–60	50,270

The energies of activation (E) for the various temperature ranges investigated were calculated from the velocity constants of the fast reactions (Table 2). The decrease in activation energy over the temperature range studied suggests that as temperature increases either a smaller input of energy is necessary to induce physicochemical changes in the virus or there is less virus present which is susceptible to heat inactivation.

The results of two experiments with the WR strain of virus at 55° are similar to those with the Lister Institute strain at 55° (Fig. 4). Clearly, strains which behave very differently in infected hosts may have similar susceptibility to heat.

DISCUSSION

It has been generally assumed that the inactivation of viruses at different temperatures is exponential (Luria, 1953). Adams (1949) found that the inactivation of bacteriophage by heat was exponential, and increased very rapidly over the 10° range from 63° to 73°. Bronson & Parker (1943) reached similar conclusions about the inactivation of myxoma virus. Fenner (1953) had similar results with two strains of vaccinia virus. He did not, however, continue his experiments long enough to reach zero infectivity; this point was estimated by graphic extrapolation of the line obtained in short-period experiments. Bourdillon (1944), working with a partially purified mouse-brain preparation of Columbia SK virus, obtained a family of inactivation curves at 49·5°, 56·5° and 63°. His results suggested that the reaction did not follow first-order kinetics, but are insufficient to allow any firm conclusions to be drawn from them. Lauffer & Carnelly (1945) and Scott & Lauffer (1946) were unable to show that influenza virus haemagglutinin was destroyed as a first-order reaction when heated. They managed to obtain a linear relationship by plotting the reciprocal of the square root of residual haemagglutinating activity against time. This relationship indicates that influenza haemagglutinin is heterogeneous in its heat sensitivity—a state of affairs readily ascertained by less circuitous methods.

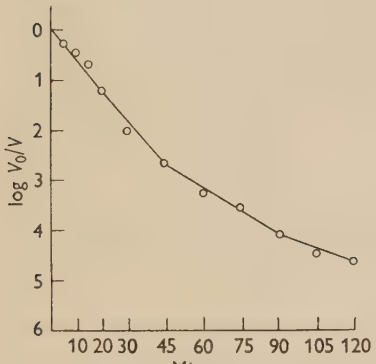

Fig. 3

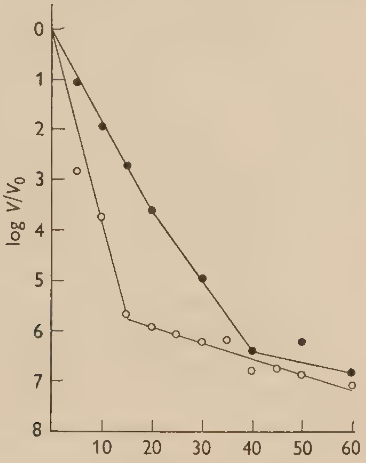

Fig. 4

Fig. 3. Inactivation at 50°: composite of two 60 min. experiments and four 120 min experiments.

Fig. 4. Inactivation of two strains of vaccinia virus at 55°. ○, Lister strain: 6 experiments; ●, WR strain: 2 experiments.

Lauffer, Wheatly & Robinson (1949) inactivated influenza virus at various temperatures in the presence of urea or formaldehyde. In only one case did they continue an experiment for as long as 30 min. They made no comment about heterogeneity of response to heat, but since the lowest titres reached in their experiments were in the region of 10^4 LD50 doses they may not have reached the level at which heterogeneity would be manifest. Lauffer & Wheatly (1951) re-investigated the problem of the alleged 3/2 order reaction with influenza haemagglutinin and concluded that the behaviour of their virus was conditioned by the presence, in even the most highly purified preparations, of a mixture of rapidly and slowly reacting haemagglutinin particles. Bachrach, Breese, Callis, Hess & Patty (1957) showed that foot-and-mouth disease virus has a heat inactivation pattern very similar to that found by us for vaccinia.

Our results suggest that vaccinia virus preparations also contain particles with two or three sensitivities to heat. It is more likely, however, that a given vaccinia virus population contains a great preponderance of particles which are rapidly inactivated and a smaller proportion of varying susceptibility to heat. This can be seen in Fig. 2. in which the resistant fraction is decreased from about 10 % at 50° to less than 0·000001 % at 60°. The values of E in Table 2 also suggest that as the temperature increases there is a progressively smaller proportion of heat-resistant particles which have to be endowed with enough energy to raise them to the activated state in which changes in molecular structure may be expected to occur.

The suggestion that virus populations are heterogeneous in various respects has been made for several animal viruses. Smith & Cohen (1956) separated influenza virus strains into rapidly- and slowly-eluting components, and obtained fractions unable to elute spontaneously from human red cells. The progeny both of rapidly-eluting and of non-eluting fractions proved in each case to be of mixed elution behaviour, i.e. similar to the original strain. Gard (1957), in a discussion on the chemical inactivation of viruses by formaldehyde, concluded that the manifest divergence from first-order kinetics of not only poliomyelitis virus but also tobacco mosaic virus and probably influenza virus was evidence of heterogeneity He put forward two theories: that heterogeneity is genetically determined, or that it is induced by the exposure to formaldehyde. He considered that the fact that genetically homogeneous strains of virus could not be obtained was evidence against the first and in favour of the second hypothesis. Although our results agree with Gard's in that resistance to inactivation is not genetically controlled, heterogeneity of various types is not excluded as a normal attribute of virus populations. Although it is possible that the coagulating action of formaldehyde renders surviving virus steadily less susceptible to other chemical actions, it is very difficult to imagine any way in which exposure to heat could make the exposed virus particles more resistant to heat.

No explanation can be offered for the fact that the inactivation rate of the resistant fraction of virus is independent of temperature over the range studied. It is possible that some light may be shed on this by studies of other methods of inactivation. We obtained curves of similar shape when vaccinia virus was exposed to large doses of γ-radiation. Whatever its explanation, there is little doubt that the pattern of inactivation of this virus, and of others (Bourdillon, 1944; Lauffer & Carnelly, 1945; Scott & Lauffer, 1946; Lauffer & Wheatly, 1951; Woese, 1956; Timm, McLean, Kupsky & Hook, 1956; Taylor *et al.* 1957) is a character of many viruses when they are inactivated by agents as different as heat, formaldehyde and ultraviolet radiation. This means that the margin between inactivation of infectivity and destruction of antigenicity may often be too small to be of practical value. It also means that, since small numbers of virus particles may resist inactivation for long periods, sensitive and stringent tests must be used to detect this minute proportion of survivors in inactivated virus vaccines. This point has recently been made by Gard and other workers in the poliomyelitis vaccine field, but it is a subject of such great practical importance that it cannot be overstressed.

I wish to thank Miss V. Finnegan and Mr J. Squires for their technical assistance; and Dr R. H. Painter for several interesting and profitable discussions.

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Activation and Thermostability of *Neurospora crassa* Tyrosinase

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SUMMARY: Tyrosinase in crude cell-free preparations from two strains of *Neurospora crassa* is markedly activated by gentle heating or by exposure to sodium ion, urea, or certain organic nitrogenous bases. The tyrosinase activity of non-activated extracts increases during assay, whereas activated enzyme displays the 'reaction inactivation' kinetics characteristic of many copper oxidases. Activation by Na^+ is antagonized by K^+ and is readily reversible. Urea activation is also reversible, but the activation due to heating appears to be irreversible. The thermostability of tyrosinase derived from strain T^S is increased greatly by Na^+ (and less effectively by K^+) and the half-life of thermal inactivation at 59° varies between wide limits, depending on the ionic environment. The relatively heat-labile tyrosinase of strain T^L shows qualitatively similar behaviour but is quantitatively different with regard to stabilization by Na^+ and K^+.

Most strains of *Neurospora crassa* produce tyrosinase during vegetative growth, and previous studies from this laboratory (Horowitz & Fling, 1953, 1956*a*, *b*) have shown that the enzyme occurs in two forms which differ markedly in thermostability. The half-lives of thermal inactivation of the two forms at 59° in 0·1 M-phosphate buffer pH 6 have been reported to be 3 and 70 min., respectively (Horowitz & Fling, 1953). Genetic analysis indicates that the thermostability property is determined by a pair of Mendelian alleles, designated as T^L (thermolabile) and T^S (thermostable) (Horowitz & Fling, 1953). The present report is concerned with 'activation' of the cell-free tyrosinases of the T^L and T^S strains and with the influence of chemical factors on the thermostability property.

METHODS

Neurospora crassa strains 4–137*a* (T^L) and 4–121 A (T^S) were grown for 4 days at 25° in the 'low sulphur' minimal medium described by Horowitz & Shen (1952), with the exceptions that the carbon source was 1·5 % glucose and ammonium nitrate was omitted. To prepare cell-free extracts, the harvested mycelium was thoroughly washed with water, ground with sand (0·5 g/g. moist mycelium), and the mixture extracted with approximately 10 vol. of water. After a preliminary centrifugation to remove sand and cell debris, the extracts were recentrifuged for at least 1 hr. in the International Refrigerated Centrifuge (*c.* 20,000 *g*) or in the Spinco Centrifuge (*c.* 140,000 *g*).

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For the experiments summarized in Table 3, the organisms were grown and the partially purified tyrosinases prepared as described by Horowitz & Fling (1953).

Tyrosinase activity was determined colorimetrically, essentially as described by Horowitz & Shen (1952), using DL-3:4-dihydroxyphenylalanine as the substrate. In this procedure, activity is measured in terms of optical density increase (expressed as colorimeter units) due to 'dopachrome' formation, determined in the Klett-Summerson photoelectric colorimeter using a no. 42 blue filter. The 'mixed' 0·1 M (pH 6) phosphate buffer used for the experiments of Fig. 1. and Table 3 was composed of 0·088 M-KH_2PO_4 and 0·012 M-Na_2HPO_4.

RESULTS

Activation of tyrosinase

Typical results showing the effect of a short heat treatment on tyrosinase activity in crude extracts of *Neurospora crassa* (T^S strain) are shown in Fig. 1. Curve *B* represents the course of the reaction observed with untreated extract, while curve *A* illustrates the activity observed with extract preheated at 59° for 1·5 min. It is evident that the short heat treatment increased the activity markedly, and it may be noted that the activated preparation shows the type of kinetics frequently observed with copper enzymes such as ascorbic acid oxidase or mushroom tyrosinase, i.e. a rapid decrease in activity during the course of the reaction, which is usually referred to as 'reaction inactivation' (Dawson & Tarpley, 1951). The unheated preparation, on the other hand,

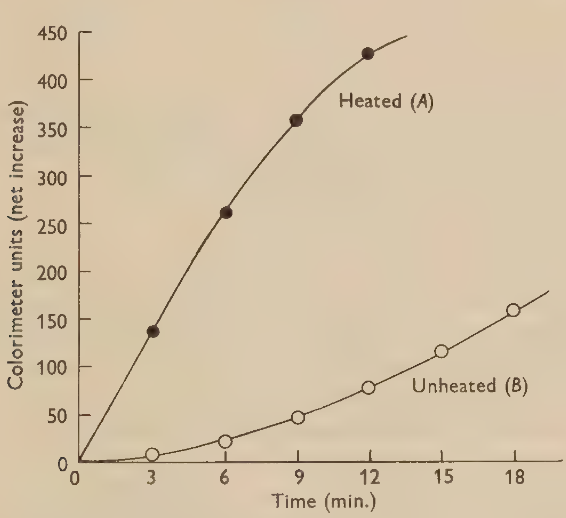

Fig. 1. Effect of preheating at 59° for 1·5 min. on the activity of T^S tyrosinase. Curve *A*: extract was diluted twofold with water, heat treated, and the activity of a 0·08 ml. aliquot measured (in 'mixed' 0·1 M-phosphate buffer pH 6). In order to facilitate direct comparison with the activity shown by 0·2 ml. of unheated extract (curve *B*), the observed increases in optical density were multiplied by 5 to give the points indicated. Curve *B*: activity of 0·2 ml. unheated extract in 0·1 M-phosphate buffer pH 6.

shows a gradual increase in activity, presumably due to activation occurring during the assay. Extracts of strain T^L showed the same behaviour.

The thermal activation appears to be irreversible. It is of interest that the activity of partially purified *Neurospora crassa* tyrosinase is usually not increased by short-term heating (Horowitz & Fling, 1953), apparently because activation occurs during the purification procedure.

It was observed that the tryosinase in both T^S and T^L extracts can also be fully activated by addition of sodium ion. The activation due to Na^+ is antagonized by K^+ as shown in Table 1. In the absence of added K^+, a relatively low concentration of Na^+ effectively activates the enzyme. With a large excess of Na^+, the reaction kinetics observed were qualitatively similar to those shown in curve *A* (Fig. 1), whereas with a large excess of K^+ a gradual increase in rate occurred. As might be expected, the kinetic curves for intermediate mixtures were complex, but in general they reflected the effect of the predominant cation. The antagonism observed between Na^+ and K^+ suggests that the Na^+ effect is reversible and this was confirmed in experiments in which a large excess of K^+ was added following activation by Na^+—the resulting depressed activity was the same as that shown by enzyme added at zero time to a buffer containing previously mixed sodium and potassium succinates in the same ratio. Tyrosinase inhibited by excess K^+ can be fully activated by gentle heating (see Fig. 1).

Table 1. *Activation of* Neurospora crassa *tyrosinase by sodium ion*

	Buffer*			
Tube no.	Na succinate (ml.)	K succinate (ml.)	Na succinate/ K succinate	Initial activity (colorimeter units)
1	4·4	—	—	132
2	0·9†	—	—	126
3	0·4†	—	—	118
4	4·0	0·4	10	99
5	3·0	1·4	2·14	61
6	1·4	3·0	0·47	36
7	0·4	4·0	0·1	28
8	—	4·4	—	21

In each case, 0·1 ml. of a water extract of T^S was mixed with buffer; after equilibration at 30° for 2·5 to 3·5 min., 0·5 ml. of 0·02M DL-3,4-dihydroxyphenylalanine was added and the activity determined during the first measurable 3 min. interval.

* pH 6·0 and 0·05M with respect to succinate.

† Supplemented with water to a volume of 4·4 ml.

Neurospora crassa tyrosinase can also be activated (reversibly) by *c.* 3M-urea and tests with tris 2-amino-2-hydroxymethylpropane-1:3-diol and triethylaminesuccinate buffers (pH 6) showed that these nitrogenous bases also effected complete activation.

Thermostability experiments

The half-lives of thermal inactivation of crude T^S tyrosinase at 59° in various pH 6 buffers are summarized in Table 2. In all instances, first-order inactivation kinetics were observed. From these data it can be seen that Na^+

Table 2. *Thermostability of crude T^S tyrosinase at 59° in various buffers at* pH 6

Exp.	Buffer*	Half-life† at 59° (min.)
1‡	Na succinate	67
2	Na succinate	47
3	Na succinate (3 parts) + K succinate (1 part)	51
4	Na succinate (1 part) + K succinate (3 parts)	34
5‡	K succinate	11·5
6	K succinate	8
7	Tris-succinate	2·5
8	Triethylamine-succinate	*c.* 1

* In Exps. 1 through 6, the buffer was 0·045 M with respect to succinate. In Exps. 7 and 8, the buffers contained 0·05 M-succinic acid and *c.* 0·09 M-tris or triethylamine.

† To obtain thermal inactivation curves, 0·5 ml. samples were heated at 59° and after cooling the samples, activity was measured as usual using 0·04 M-Na succinate as the assay buffer (in Exp. 7, tris-succinate buffer was used for assay).

‡ Extract dialyzed (at *c.* 5°) against water for 5 hr.; the tyrosinase was activated by this treatment and the dialyzed extract showed a slight pink colour.

not only activates T^S tyrosinase but also stabilizes the enzyme markedly with respect to thermal inactivation. On the other hand, in the presence of certain nitrogenous bases the enzyme is fully activated but is very heat labile. In comparison with the organic bases, K^+ has some stabilizing influence and judging from Exp. 4 it appears that Na^+ can significantly increase the half-life in the presence of relatively large amounts of K^+. Preactivation of T^S tyrosinase by heating at 59° for 1·5 min. did not appreciably affect the thermostabilities in sodium, potassium, and triethylamine-succinate buffers.

Table 3. *Thermostability of partially purified T^L and T^S tyrosinases at 59° in sodium and potassium phosphate buffers at* pH 6

			Half-life at 59° (min.)†	
Exp.	Buffer	Conc.* (M)	T^L	T^S
1	Water	—	*c.* 0·5	*c.* 2
2	Sodium phosphate	0·04	—	51
3	Sodium phosphate	0·08	3·5	67
4	Sodium phosphate	0·16	—	140
5	Sodium phosphate	0·80	19.0	120
6	Potassium phosphate	0·04	—	7
7	Potassium phosphate	0·08	*c.* 0·5	14
8	Potassium phosphate	0·16	*c.* 1	28
9	Potassium phosphate	0·40	3	—
10	Potassium phosphate	0·80	6	75

For these experiments, the organisms were grown and the tyrosinases extracted as described by Horowitz & Fling (1953). The enzymes were purified by their procedure and then dialyzed against 0·05 M-potassium phosphate pH 6.

* Final concentration of added phosphate; note that due to the dialysis, *c.* 0·01 M-potassium phosphate was introduced with the enzyme preparation (see †).

† To obtain thermal inactivation rates, 0·5 ml. samples (consisting of 0·1 ml. enzyme and 0·4 ml. buffer) were heated at 59° and after cooling the samples, activity was measured as usual using 'mixed' 0·1 M-phosphate pH 6 (see Methods) as the assay buffer.

Crude T^L tyrosinase showed qualitatively similar behaviour with respect to stabilization by Na^+, i.e., typical half-lives observed in sodium and potassium succinate buffers were 3 and 0·5 min., respectively.

The effects of sodium and potassium ions on the thermostability of partially purified T^L and T^S tyrosinases in phosphate buffer pH 6 are shown in Table 3. As with the crude enzymes, the thermostability of the fractionated preparations was greatly enhanced by Na^+. It is evident that K^+, at comparable concentrations, was much less effective, and also that the half-life of both T^L and T^S tyrosinases can be appreciably lengthened by increasing the buffer concentration.

DISCUSSION

Activation of tyrosinase has been previously observed in preparations from grasshopper eggs (Bodine, Tahmisian & Hill, 1944), *Drosophila melanogaster* larvae (Ohnishi, 1953), and Drosophila adult flies (Horowitz & Fling, 1955). Other reports have also suggested the existence of a 'protyrosinase' which can be activated by heating in *Neurospora crassa* (Horowitz & Shen, 1952; Schaeffer, 1953; Fox & Burnett, 1956) and in the ascomycete *Gelasinospora tetrasperma* (Boulter, 1957). Activation of tyrosinase and other copper oxidases can also be effected by various chemical agents, e.g., the grasshopper enzyme (Bodine *et al.* 1944) is activated by urea and certain detergents, while ascorbic acid oxidase (Frieden & Maggiolo, 1957) is activated by a variety of substances (proteins, amino acids, thyroxine analogues, etc.) which in general possess the ability to chelate copper. Al^{+++} and several divalent cations also appear to activate the latter enzyme (Frieden & Maggiolo, 1957). The present experiments indicate that Neurospora tyrosinase is activated by certain organic nitrogenous bases, which may act through interaction with the copper moiety of the enzyme, and also by Na^+. In connexion with the sodium ion effect, it is of interest that a significant binding of Na^+ (and K^+) by several proteins has recently been observed (Carr, 1956; Lewis & Saroff, 1957) and that the activity of a number of enzymes is markedly enhanced by Na^+ or K^+ (e.g. salivary amylase, Schneyer, 1952; Rotini & Galoppini, 1952–53; β-galactosidase, Landman, 1957; pyruvic phosphoferase, Kachmar & Boyer, 1953). Considering the diversity of agents which share the ability to activate copper oxidases and the complex nature of these catalysts (Mallette & Dawson, 1949; Mason, 1956) it seems likely that several different mechanisms may be involved in the activation phenomenon. This conclusion is supported by the observation that thermal activation of Neurospora tyrosinase appears to be irreversible, while the activation by Na^+ or urea is readily reversible.

Stabilization of enzyme activity, with respect to thermal inactivation, by various cations has been frequently observed (e.g. with salivary amylase, Schneyer, 1952, Rotini & Galoppini, 1952–53; and with trypsin, Crewther, 1953), and the present data demonstrate that the thermostability of *Neurospora crassa* tyrosinase also is strongly influenced by the ionic environment. In this regard, it should be noted that Na^+ activates the enzyme and exerts a marked stabilization effect, whereas tris (buffer) and triethylamine, which also activate, do not protect against thermal inactivation.

Thus far, attempts to demonstrate significant differences between the T^S and T^L *Neurospora crassa* tyrosinases, other than with respect to thermostability, have been unsuccessful (Horowitz & Fling, 1956*a*). Both enzymes appear to be similar with regard to the activation phenomenon, but they do show quantitative differences with respect to stabilization by Na^+ and K^+. It is probable that these differences are due to modifications in the structural organization of the enzyme in the two strains.

This work was done while one of us (H. G.) was a Public Health Service Research Fellow of the National Cancer Institute, United States Public Health Service; on leave (1956–57) from the Department of Microbiology, Western Reserve University School of Medicine, Cleveland, Ohio, U.S.A.

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Some Unusual Chrysophyceae Studied in the Electron Microscope

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SUMMARY: Three members of the Chrysophyceae are described, the general morphology being studied with the light microscope, but the details of the scale and bristle structure with the electron microscope. *Mallomonas ouradion* sp.nov. is at present unique in having several movable bristles on each scale. *Chrysosphaerella brevispina* Korshikov is shown to include *C. rodhei* Skuja. The peculiar bristle base is elucidated. This is the first record for the genus in Britain. *Synura sphagnicola* is shown to differ in several respects from other species of Synura. In all three genera, the chromatophore, which has been described as double, is shown to be single but strongly lobed. Replicas of the intact armour of *S. echinulata* are shown.

The classification of the species of Mallomonas, Chrysosphaerella and Synura has used the minute silica scales and bristles with which these organisms are covered, in addition to the general morphology of the cell itself. The sculpturing of the scales is, for the most part, beyond the resolution of the light microscope, so that classification based on these features was necessarily somewhat vague. The high resolution of the electron microscope has permitted the smallest structural details of the scales to be seen, and the use of replica techniques has enabled arrays of scales on complete organisms to be studied and also the front and back of the scales to be distinguished (Harris & Bradley, 1957). As a result, differentiation between species has become much easier, making possible a fuller and clearer classification and the identification of many new species.

METHODS

Collection of organisms

Samples of pond water were collected and the organisms concentrated by centrifuging at about 2000 rev./min. A drop of concentrate was then examined in the light microscope and the living organisms identified as nearly as possible and drawn. Cytological observations were made mainly on living material, though, when necessary, they were confirmed with fixed material. The methods of fixation used were either one half-minute exposure to osmic acid vapour or the addition of a drop of the algal concentrate to a minute drop of osmic acid solution or strong formalin previously placed on a slide. The latter method was used where there was a covering of mucilage on the colonies, as the fixative seemed to penetrate the mucilage better when used in this way, tending to prevent the colonies from breaking up. No method of fixation was wholly satisfactory. For examination of scales and bristles, preparations were made by allowing a drop of the concentrate to dry on the slide. This could be examined with the light microscope either dry or mounted in resin of high refractive index, or used after further treatment for examination in the electron microscope.

Electron microscopy

The specimens were examined in the electron microscope, both directly and by means of carbon replicas (Bradley, 1954*a*). Preparative techniques have been described in detail elsewhere (Harris & Bradley, 1957), so that only a brief outline need be given here. In the case of direct examination, the slide on which the organisms had been mounted was coated with a layer of carbon to act as a support film (Bradley, 1954*b*). This deposit was then backed with plastic, and the combined film, with the organisms embedded in it, was floated on to water and cut into small pieces. These were mounted on specimen support grids, and the plastic backing washed away with a suitable solvent. The organisms remained within the carbon film and were ready for examination in the electron microscope.

Where replicas were required, a plastic-backed carbon film containing the organisms was prepared as above, and then floated on 50% (w/v) hydrofluoric acid for 15 min. to dissolve the silica scales and bristles. Organic matter was removed by means of a solution of potassium permanganate in sulphuric acid. The plastic backing was washed away as before, and the carbon 'shell' which constituted the final replica shadowed and examined in the electron microscope.

These two methods of examination were used because it was found that the morphology of the scales is most clearly visualized by combining the information provided by replicas and by direct examination. The reason for this is that the mechanism of contrast formation by the scales themselves is completely different from that of replicas. In the cases of the scale itself, internal structure is shown and punctate markings more clearly seen; with a replica, a marked three-dimensional effect is produced, inner and outer surfaces of the scales may be differentiated, and a more easily interpreted picture is obtained.

Electron micrographs of replicas are printed as negatives so that it appears as if the scales are illuminated obliquely by white light.

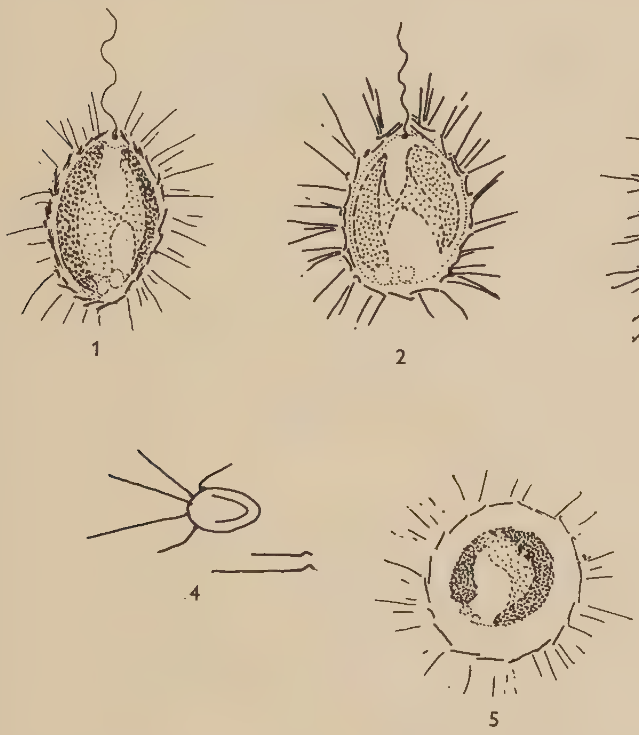
RESULTS

Mallomonas ouradion sp.nov.

This is a new species and is illustrated in Figs. 1–5, Pl. 1, figs. 1–7 and Pl. 2, fig. 8.

Diagnosis

Cellula ovoides, semper ferme 20–25 × 10–15 μ., setis multis gracilibus contacta. Chromatophorum solum prope in lobos duo divisum, colore subpallido. Flagellum longius quam corpus, squama ovalis, semper ferme 5–7 × 3–4 μ., scutum et margo praesens sed cupola absens; scutum externe papillis parvis tectum; margo latus politus; squama tota interne levis, cavulo parvo in parte remota a margine verisimiliter poris minutis indistinctis plerumque sparsa. Squama setas tres inter quinque ferens, basis setae lata, subter inflecta vel uncinata, truncus setae rectus, 4–12 μ. longe, profunde, sulcatus; apex setae in cuspides duo equales furcatus.



Figs. 1–5. *Mallomonas ouradion.* Fig. 1. Individual; ×1000. Fig. 2. Individual replacing two anterior scales; ×1000. Fig. 3. Individual replacing all scales; ×1000. Fig. 4. Scale and two bristles; ×2000. Fig. 5. Individual in spherical condition.

Cell ovoid, typically 20–25 × 10–15 μ., covered all over with numerous delicate bristles. Chromatophore single, deeply divided into two lobes, rather pale in colour. Flagellum over body length. Scale oval, typically 5–7 × 3–4 μ. having shield and flange but no dome; shield covered on outer side with small papillae each pierced by a small hole; flange broad, smooth; underside of whole scale smooth but with slight hollow at end opposite flange; most of underside probably covered with minute indistinct pores. Bristles 3 to 5 per scale; base of bristle broad, sharply bent or hooked, shaft straight, 4–12 μ. long, smooth, deeply grooved, enlarged and forked into two equal parts at the tip.

The name *ouradion*, a fish tail, refers to the shape of the bristle tip.

For terminology see Harris & Bradley, 1957.

Occurrence. Common near Reading, May to October in peaty pools, usually with *Sphagnum*, occurring with *Synura sphagnicola* Korsch. It is easily overlooked because of its small size.

Discussion of Mallomonas ouradion

The chromatophore of *Mallomonas ouradion* is similar to that found in many species of *Mallomonas* (Harris, 1953). The two rather broad lobes, situated in the peripheral protoplasm and curving round the cell, look at first sight like two separate chromatophores. The connexion between the lobes is delicate and pale, while the lobes themselves are a distinct brownish green colour (Figs. 1–3 and 14). The nucleus is enclosed by the lobes at the anterior end of the cell and is distinct. A leucosin body is usually present between the lobes in the rear part of the cell and, as in all species of *Mallomonas*, varies in size at different times.

Mallomonas ouradion is at present unique in that the scales bear from three to five bristles, and not a single bristle as in all other known species of the genus (Fig. 4 and Pl. 1, fig. 3). The bristles have a base which is unusual in being expanded like a spade. It is sharply bent and fits under the shield into a slight hollow (Pl. 1, figs. 2, 5 and 6). The bristles are expanded at their free ends into the shape of a fish tail, a shape characteristic of this species alone.

The presence of several bristles on a scale raises the question whether this species should be considered the type of a new genus. We refrained from suggesting this classification because *Mallomonas ouradion* seems to be a typical *Mallomonas* in its other characteristics.

One scale shown in Pl. 2, fig. 8, is unlike the others in being without papillae. This scale, partly overlapped by two others and seen near the right of the figure, must be showing its outer surface since the characteristic marginal flange is present. No papillae can be seen except three or four possible vestiges on the lower margin. We regard this as an immature scale replacing one that has been shed. We know already that scales are shed and replaced in *Mallomonas* individuals (Harris, 1953). Fig. 2 shows one individual in which the anterior scales appear to have been replaced before the old ones have been shed, and Fig. 3 shows an unusual individual in which two complete layers are present. If our interpretation of this smooth scale is correct, then it has a special interest in suggesting that the scales are first produced as delicate plates of full size and that the external sculpture is added afterwards.

No cyst has been seen, but *Mallomonas ouradion* can pass into a peculiar state in which the organism is spherical and the protoplasm is shrunk away from the scale armour, forming an interior sphere (Fig. 5). This condition was not a transient one; large numbers of these spheres were found, and they persisted in the bottle for several days without change. Gradually they disappeared, presumably through death.

History of the genus Chrysosphaerella *Lauterborne*

Chrysosphaerella has been recorded from a few lakes in various parts of Europe and the United States. The type is *C. longispina* Lauterborn (1896, 1899). It was recorded with figures by Smith (1933), redescribed by Korshikov (1941–42) and recorded with figures by Skuja (1948). The record by Conrad (1941) appears to have been *C. brevispina* Korsch. not *C. longispina*

Lauterborne. Two other species besides *C. longispina* have been recorded, but our observations indicate that they should be united. The earlier is *C. brevispina* Korshikov (1941–42), and the later *C. rodhei* Skuja (1948). The latter has also been described by Fott & Ludvik (1956) from scales and bristles found in peat and examined in the electron microscope, but they did not see any living material.

Chrysosphaerella brevispina *Korshikov, emend.*

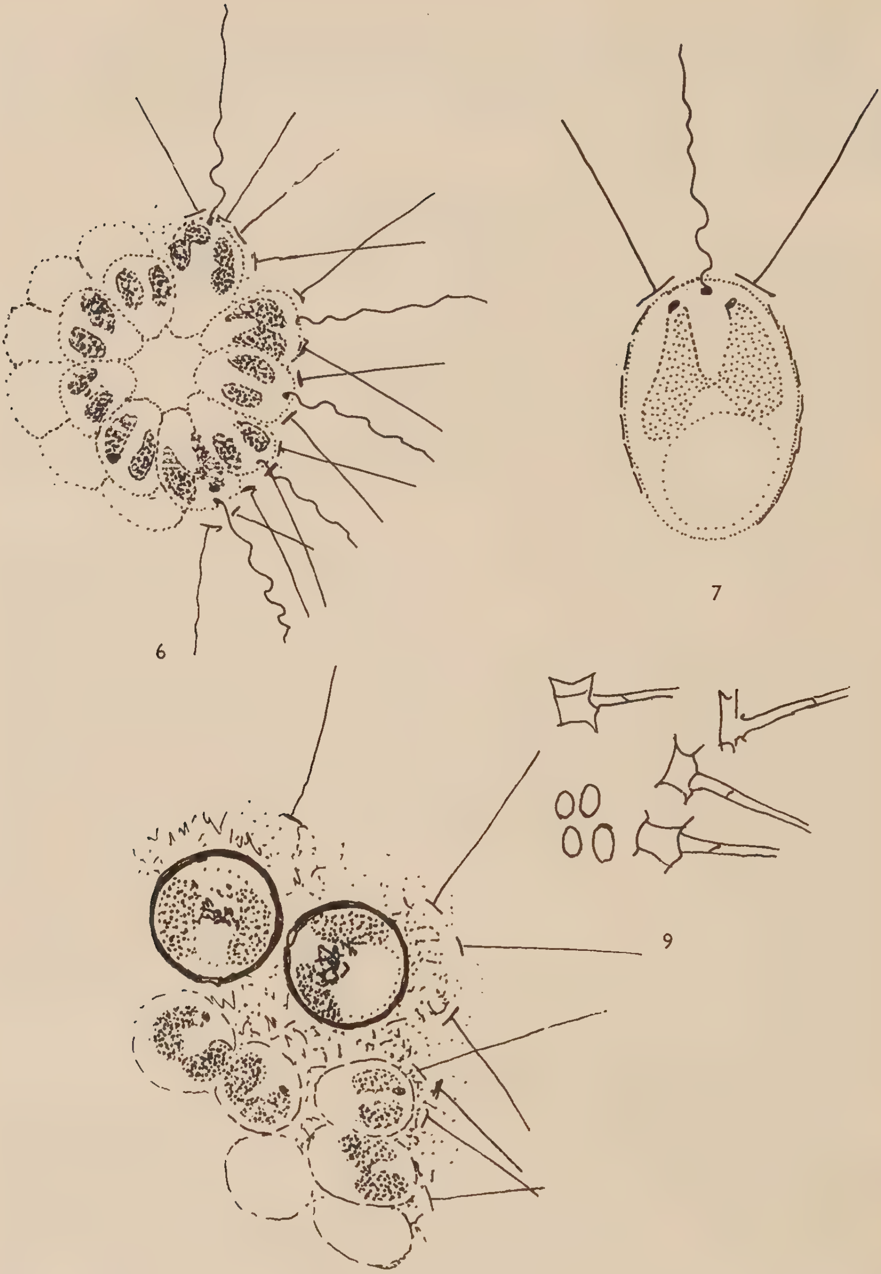
This organism is illustrated in Figs. 6–9 and Pl. 2, figs. 9–13.

Light microscopy. Chrysosphaerella brevispina is a colonial organism with ovoid cells. These possess delicate scales and a few long bristles. The colony is usually coated with mucilage which takes up methylene blue and resists penetration by indian ink. A number of loose scales appear to be embedded in the mucilage. In a broken colony, we were able to observe that the centre is hollow, the cells not being joined by strands of protoplasm (Fig. 6).

Isolated living cells have a membrane which seems to be covered all over with exceedingly delicate scales, although their arrangement cannot be seen (Fig. 7). It is impossible in a living colony to see whether the scales surround each cell or are confined to the outer free surface of each cell. In both isolated cells and colonies, bristles may be seen, apparently arising from small plates situated just outside the cell membrane. We did not succeed in obtaining any successfully fixed cells in which the scales retained their original position. We think that the difficulty may have been caused by mucilage slowing down the penetration of fixative. The cells always disintegrated as they died. Thus the only indications as to the arrangement of the scales are such as can be seen on the living cell. There are up to seven bristles attached to each cell. These may be most easily counted in dried material, though their arrangement is disorganized. Fig. 6 shows a group of scales and bristle bases as they appear when they have been mounted in resin. The bristle tips look simple at this magnification.

The chromatophore of *Chrysosphaerella brevispina* differs, in our opinion, from any description hitherto given. Normally the chromatophore is single but deeply divided into two lobes. The connexion between these lobes is narrow and exceedingly delicate so that, in many positions, the lobes look like two separate chromatophores (Fig. 7). A refractive spot may be seen in the extreme anterior tip of one or both lobes. At certain times in the life cycle of the organism, two small separate chromatophores may be seen in a cell. Each of these is divided into two lobes by a slight constriction. Cells with these chromatophores, and others with normal chromatophores, were found side by side in the same colony (Fig. 6), both in early March, when the species was first found, and in April, at the time of cyst formation. We believe that these small chromatophores are the product of chromatophore division either in preparation for cell division, or for the formation of binucleate cysts such as are to be seen in many of the Chrysophyceae, particularly in *C. longispina* Skuja (1948). Two cysts were found 11 μ. in diameter (Fig. 8).

Electron microscopy. The examination of *Chrysosphaerella brevispina* in the



Figs. 6–9. *Chrysosphaerella brevispina* Korsch. Fig. 6. Broken colony; × 1000. Fig. 7. Individual cell; × 2000. Fig. 8. Part of colony including two cysts; × 1000. Fig. 9. Scales and bristles; × 2000.

electron microscope elucidates what has been seen in the light microscope and adds much to our knowledge. The replicas show that the inner and outer surfaces of the scales are different (Pl. 2, figs. 10, 11). The outer surface shows a conspicuous row of pits just inside the margin, but the inner surface shows only a wavy fold along the inner edge of these pits. The direct electron micrograph shows both features superimposed; in some scales the pits, but in some the edge of the fold, are more clearly marked (Pl. 2, fig. 11).

The bristle is hollow, with two prongs at its tip, one usually longer than the other (Pl. 2, fig. 13). At this high magnification something of the structure of the bristle wall can be seen; its outer layers appear to be flaking off. The base, from which the bristle arises, could be described as bobbin-like, or as two concave disks joined by a hollow tube on which the bristle is placed excentrically. The hollow tube of the base is continuous with the hollow bristle (Pl. 2, figs. 9, 12). The rim of the outer disk bends back to a greater or lesser extent. This turned-back rim catches the light and is the only part of the bristle to show when the living cell is examined in the light microscope. It is seen as the single bright refractive plate already described.

Occurrence. We have found *Chrysosphaerella brevispina* in four ponds near Reading, all on acid soils but not associated with *Sphagnum*. It was found in March and April, in the cold spring of 1956, sometimes under ice. It encysted in April. It was not found at all in the mild spring of 1957.

Discussion. Skuja differentiated *Chrysosphaerella rodhei* Skuja from *C. brevispina* Korshikov on the character of the chromatophore and of the bristle base. The chromatophore described and figured by him appears to us to be the normal bilobed chromatophore which we found in *C. brevispina*. The fact that he speaks of two chromatophores would be explained if, as we think likely, he had missed the small and delicate connexion between the two lobes. The chromatophores described by Korshikov as 'biscuit-shaped' resemble very closely the chromatophores we found present in some cells at certain times, and which we believed were the result of division. If we are right, the differences between the chromatophores described by these two authors are not specific but developmental.

Although Skuja states that the bristle base of *Chrysosphaerella rodhei* differs from that of *C. brevispina* his figures do not show any difference beyond the normal variation we found within the species we have examined.

The two cysts we found are smaller than those described by Skuja being 11 μ. in diameter instead of his 13–15 μ. We do not think that this difference is significant. The range of variation in cyst size in the Chrysophyceae may be considerable within a single species.

In our opinion the species *Chrysosphaerella brevispina* Korshikov and *C. rodhei* Skuja are the same, and the name *C. brevispina* has priority.

The scales and bristles examined in the electron microscope by Fott & Ludvik (1956) agree very closely with ours. There are, however, certain differences which should be mentioned. The outer part of the bobbin in their photographs is cup-shaped, and lacks the turned-back rim which appears in ours. The extent of this turned-back rim varies considerably in our electron micrographs though in the seven we have studied, none was precisely like Fott's figure. Fott also describes the bobbin as being screw-like. We think that this appearance in his figures is due to two foreign bodies touching the bobbin and distorting its outline. We believe that the species he described is *Chrysosphaerella brevispina.*

The species described by Conrad (1941) as *Chrysosphaerella longispina* appears to be *C. brevispina.* His photographs of the scales and bristles, and also his measurements, agree with ours and not with *C. longispina* Lauterborn. His specimens were found in spring, like *C. brevispina* while, so far, *C. longispina* has only been found in autumn.

Description of the chromatophores of the genus Synura *Ehrenbg.*

Synura is usually described as having two chromatophores but we have found the chromatophores to be single and deeply divided into two lobes in all the species which we have examined. The connexion between the lobes is so narrow and delicate that, unless the cell is favourably placed, it is invisible (Figs. 14–17).

Synura sphagnicola *Korshikov* (synonym: *Skadowskiella sphagnicola*)

This organism is illustrated in Figs. 10–13, 16, and 17; Pl. 3, figs. 14–22; Pl. 4, figs. 23, 24).

Light microscopy. The light microscopy of *Synura sphagnicola* has been fully described by Korshikov, and only in a few points do we wish to add to or modify his description. It is a species which differs in a number of ways from all other well-known species, and this is an aspect which we want to emphasize.

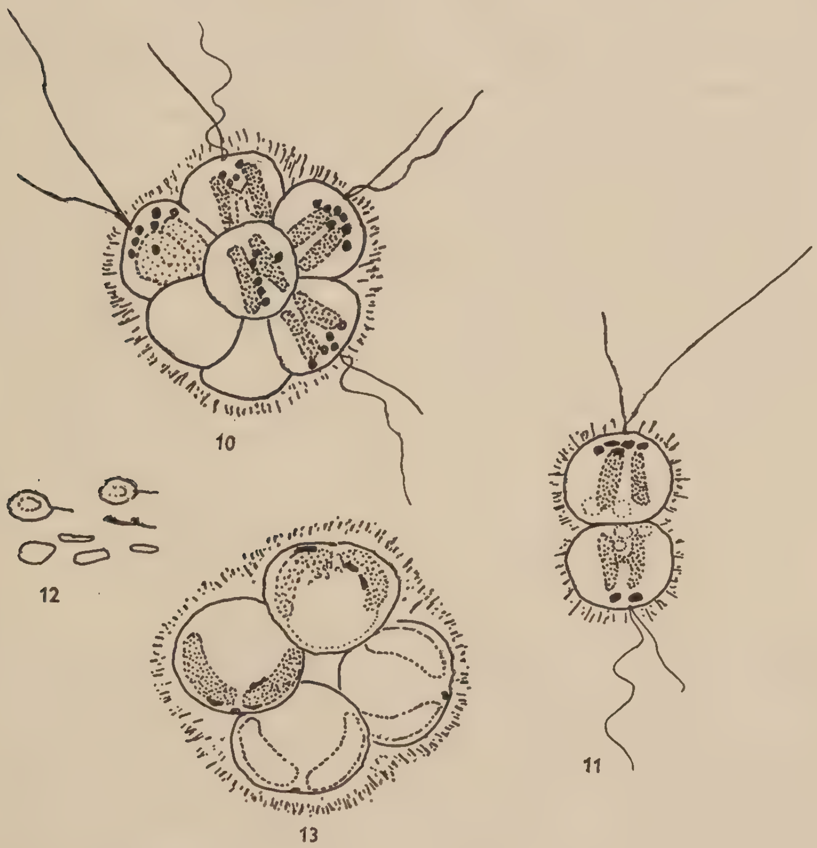
(1) It is the only known species to have red droplets. These red droplets, whose nature is not known, are situated at the anterior end of the cell. They are not part of the chromatophore and cannot be called stigmas. They may be few or many, sometimes so numerous as to coalesce and form a red patch. It is possible that they may at times be absent. They disappear suddenly, almost explosively, as the organism dies. After fixing in osmic acid, they persist for a short time, then gradually fade and disappear. They are also present in the cyst, where they appear as red disks just inside or outside the chromatophore lobes, and in this position they are inconspicuous (Figs. 10, 11 and 13).

(2) The two flagella are notably different in length, one being one-half to two-thirds the length of the other, whereas, in most of the well-known species of *Synura*, the flagella are nearly but not quite equal (Petersen, 1918; Korshikov, 1941–42; Manton, 1955).

(3) The cells forming the colony lack the protoplasmic stalks characteristic of other known species. The cells are in contact laterally but not joined centrally in any way. This may be seen when a colony is broken, and also in a colony with four cysts (Fig. 13).

(4) The chromatophore is composed of two lobes which lie side by side in the middle of the cell, joined by a narrow strip placed at right angles to them (Figs. 16, 17). In all other known species of *Synura*, the chromatophore is placed near the peripheral cytoplasm and its lobes partially envelop the cell

contents (Fig. 15). In the cyst of *S. sphagnicola*, as in all other species we have seen, the chromatophore lobes lie one each side of the pore in the outer protoplasm. The connexion between the lobes is rarely visible in the cyst (Fig. 13). Figs. 10 and 11 show the chromatophore in various positions and closely correspond to Korshikov's figures (reprinted Huber-Pestalozzi, 1941, p. 138) but Korshikov has not seen the inconspicuous connexion between the chromatophore lobes and has described the organism as having two chromatophores.



Figs. 10–13. ***Synura sphagnicola.*** **Fig. 10. Small colony; × 1000. Fig. 11. Colony of two cells; × 1000. Fig. 12. Group of scales, one seen edgewise; × 2000. Fig. 13. Colony of four cysts; × 1000.**

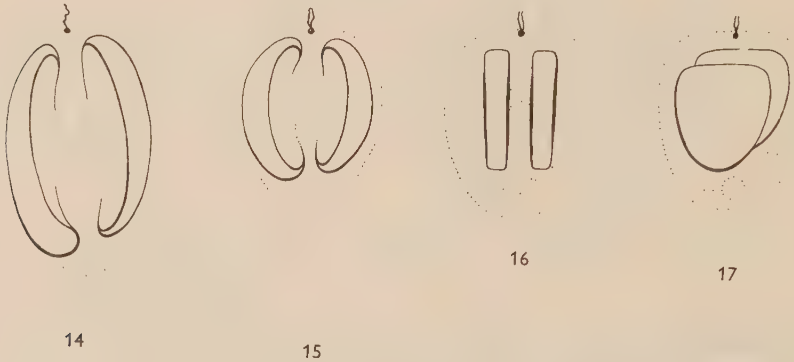
(5) In our experience, the colonies are surrounded by mucilage, which may vary in extent from a thin covering to a broad sheath in which refractive particles and scales are embedded. This zone takes up methylene blue and resists penetration by indian ink.

(6) The scales do not form a close armour as in most species of *Synura*, but appear to be set on end and point outwards.

(7) We found four cysts (Fig. 13), all of which are broader than long and

flattened round the pore. They differ thus from the cyst shown by Korshikov, which is spherical. All other species of *Synura* which we know have spherical cysts.

Electron microscopy. The scales of *Synura sphagnicola* when examined in the electron microscope show a large variety of shapes. The majority are oval, each with a fairly long spine; some are narrow with a shorter spine or none, while others are spathulate with curled edges. Intermediates are found between all these types, and they correspond to the variants found in other species (Manton 1955; Petersen & Hansen, 1956; Fott & Ludvik, 1957; Pl. 3, figs. 14–20, 22). The basis of all the broader scales is a perforated plate flat on the inner surface, but with a thickened margin on the outer side. The hollow spine, which arises just inside the margin at one end, has four small teeth at the tip (Pl. 3, fig. 21). These scales resemble those of *S. spinosa* very closely, but in *S. spinosa* the thickened margin does not continue all round the scale and there is reticulate thickening at the bristle end of the scale, this not being present in *S. sphagnicola*. The general appearance of the two organisms is so different that it should always be possible to separate them without depending on the scale structure.



Figs. 14–17. Diagrams of chromatophores. Fig. 14. *Mallomonas ouradion.* Fig. 15. *Synura echinulata.* Figs. 16, 17. *S. sphagnicola.*

Scale arrangement. Plate 4, fig. 23, is taken from a cell of *Synura sphagnicola* fixed in osmic acid vapour. A disordered ring of scales can be seen, and, in the middle, a patch of bare protoplasm. Plate 4, fig. 24, shows a similar group taken from the edge of a colony. We were unable to obtain an ordered array of scales, such as may be seen in other species of *Synura* (Pl. 4, figs. 25, 26). This was probably due to the fact that the fixative, whether a vapour or a liquid, penetrated the mucilage slowly, and the scales became detached before the organism had been killed. It is also possible that the scales never form a cohesive armour, as in other species, and are more easily and rapidly detached from the cell membrane.

Occurrence. Synura sphagnicola has been found frequently in acid ponds in the vicinity of Reading. It occurs in shallow pools associated with *Sphagnum* and in deeper ponds without *Sphagnum* and has been found both in woods and in the open. It usually occurs in summer, from May onwards, and persists even during warm weather when the ponds are drying up. It was found also in January and February of 1957 in a pond where it had not occurred during the previous summer. Possibly its occurrence was connected with the unusually mild winter.

Discussion. Synura sphagnicola differs from all other species of *Synura* both in the morphology of the cells and in the way in which they are united into colonies. The presence or absence of mucilage seems to be variable. We have always found it to be present in our material, and we have examined material in several years, in many different months, and from many localities. Fott & Ludvik state that mucilage is nearly always present, Korshikov does not find it, and Jane informs us that he has demonstrated its absence on several occasions. *S. sphagnicola*, however, is not the only species of *Synura* which may possess mucilage. We have, at times, demonstrated mucilage around colonies of *S. petersenii*, though usually none is visible in this species. Fott & Ludvik (1957) gives it as their opinion that *S. sphagnicola* and *Syncrypta volvox* Ehrenbg. are identical, that *S. volvox* is inadequately described and therefore the name *Synura sphagnicola* should remain and include the two synonyms. We are in entire agreement with this argument. Another incompletely described organism may be synonymous with *S. sphagnicola*, namely *Volvochrysis xantha* Schiffer (Huber-Pestalozzi, 1941), figures of which look very like *S. sphagnicola*. However, the description is different, but this may be discounted as it hardly agrees with its own figures.

Synura echinulata *Korshikov*

This organism is illustrated in Fig. 15 and in Pl. 4, figs. 25 and 26. Two replicas of the intact scale armour of *Synura echinulata* Korsh. are shown in Pl. 4, figs. 25 and 26. No replicas have yet been published, so far as we know, of the intact armour of any species of *Synura*. These replicas supplement the information given by Petersen & Hansen (1956) with their beautiful direct electron micrographs of *Synura* species. It is also interesting to compare them with the disordered array of scales which we obtained for *Synura sphagnicola* by the same technique.

The authors would like to thank Professor F. Jane, Holloway College, for helpful suggestions, Professor J. M. R. Cormack, University of Reading, for the Latin translation of the diagnosis of *Mallomonas ouradion*, Miss P. E. Rush of the A.E.I. Research Laboratory for her help in the preparation of electron microscope specimens, Professor T. M. Harris, F.R.S., University of Reading, for help throughout the work, and Mr M. Twyman, Fine Arts Department, University of Reading, for the diagrams, Figs. 14–17. One of the authors (D.E.B.) would also like to thank Dr T. E. Allibone, F.R.S., Director of the Research Laboratory, Associated Electrical Industries Ltd., for permission to publish this paper.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. *Mallomonas ouradion*, direct electron micrograph of a scale; × 18,000.

Fig. 2. *M. ouradion*, direct electron micrograph of a bristle; × 13,000.

Fig. 3. *M. ouradion*, shadowed carbon replica of a scale with five bristles, one of which has the tip instead of the base towards the scale; × 3200.

Fig. 4. *M. ouradion*, direct electron micrograph of a bristle tip; × 21,000.

Fig. 5. *M. ouradion*, direct electron micrograph of a bristle base; × 21,000.

Fig. 6. *M. ouradion*, shadowed carbon replica of underside of scales showing how bristles are attached; × 8000.

Fig. 7. *M. ouradion*, carbon replica of outer surface of a scale and also a bristle; × 6500.

PLATE 2

Fig. 8. *Mallomonas ouradion*, carbon replica showing one immature and several normal scales; × 10,000.

Fig. 9. *Chrysosphaerella brevispina*, direct electron micrograph of bristle base; × 15,000.

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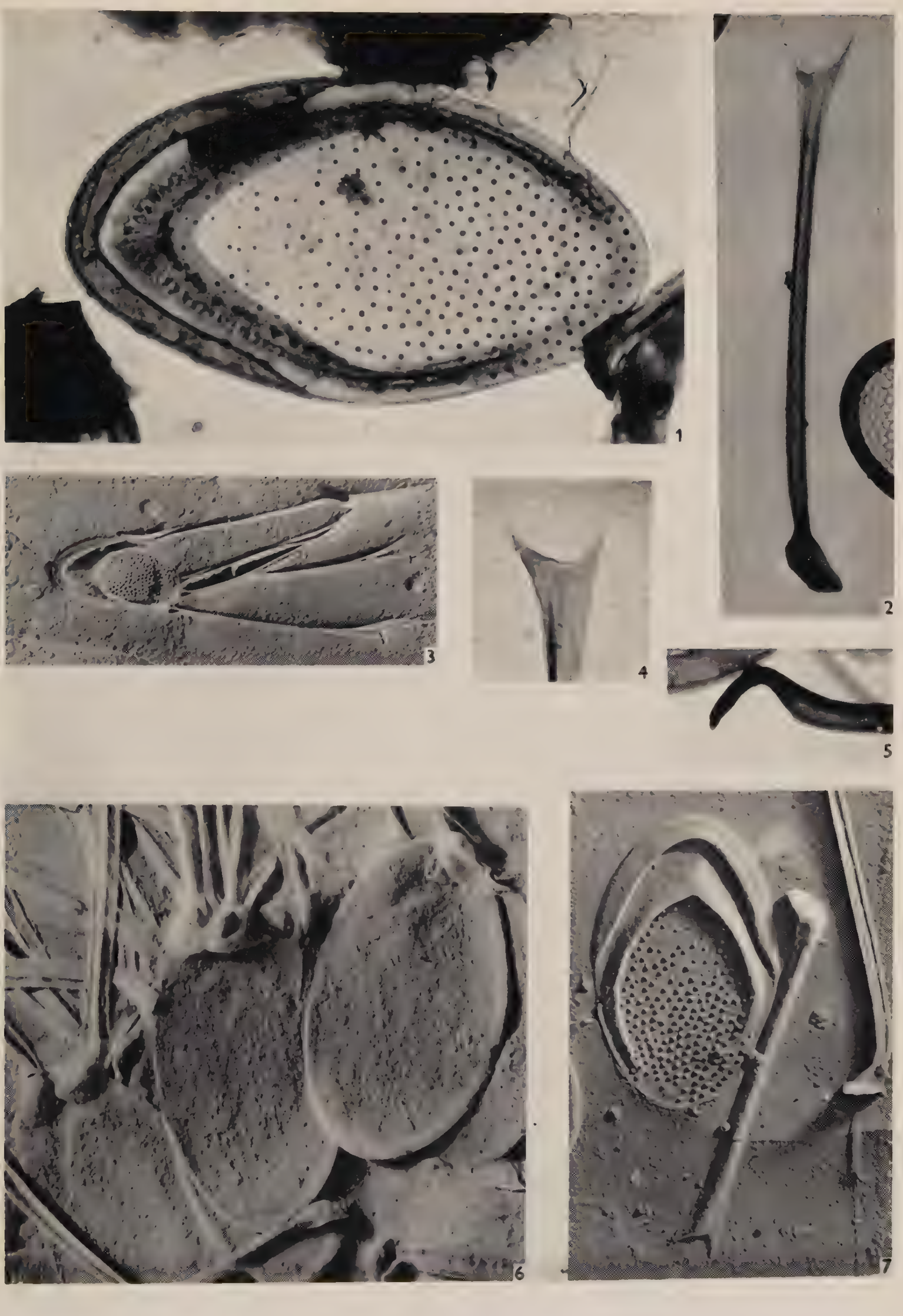

K. Harris & D. E. Bradley—Unusual chrysophyceae studied in electron microscope. Plate 1

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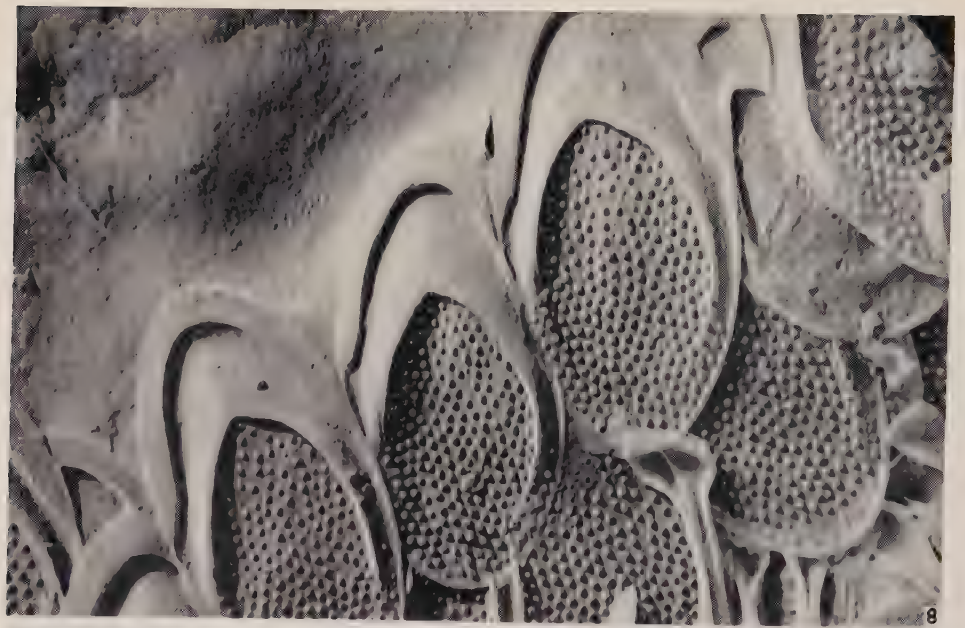
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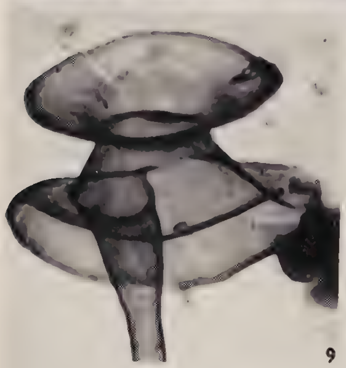
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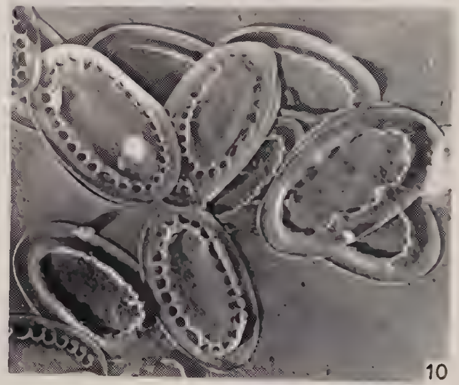
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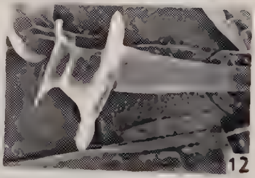
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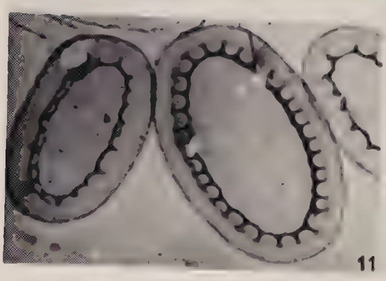
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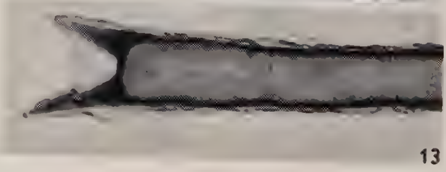
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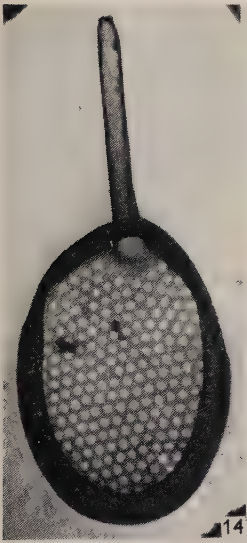
K. Harris & D. E. Bradley—Unusual chrysophyceae studied in electron microscope. Plate 2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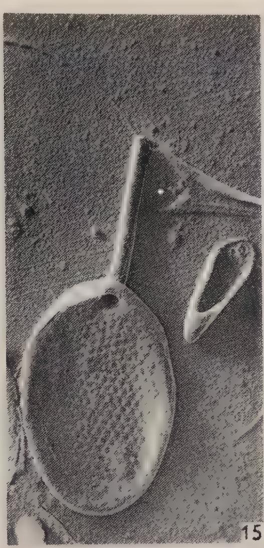
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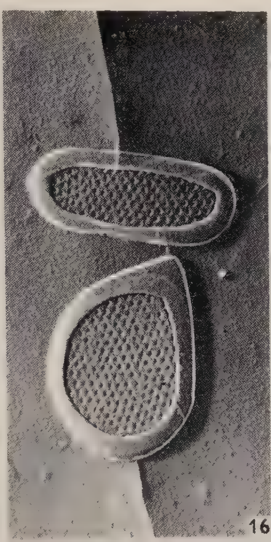
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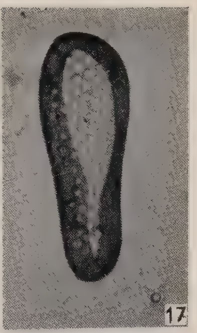
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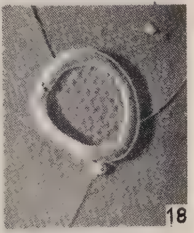
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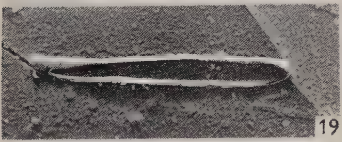
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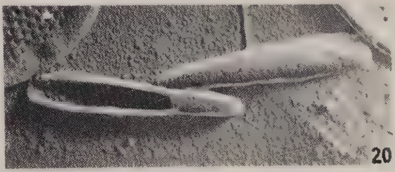
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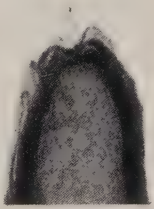
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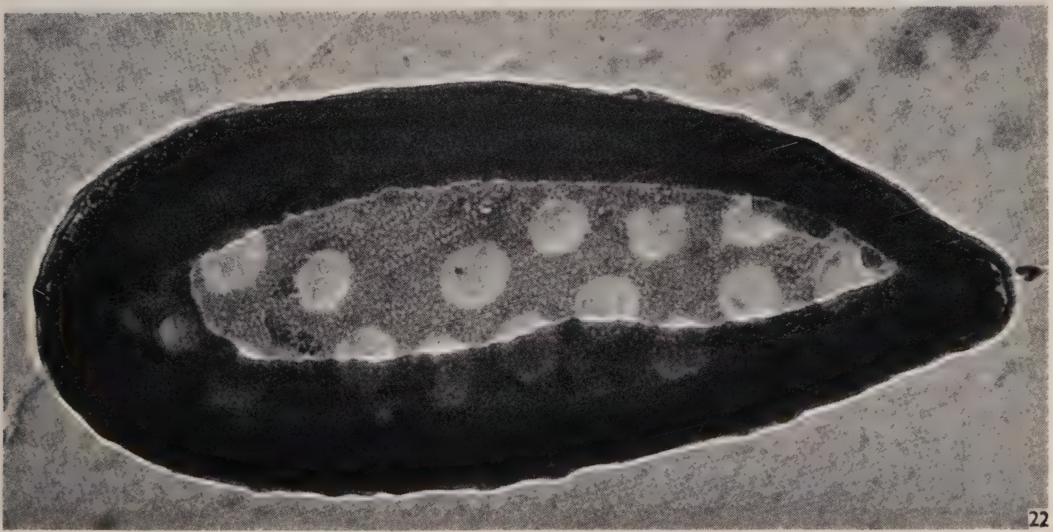
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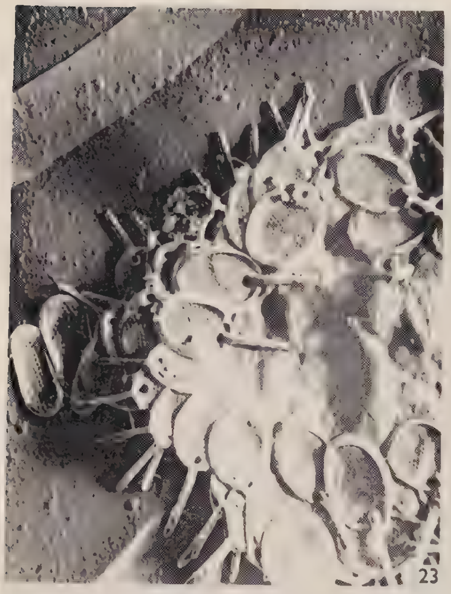
K. Harris & D. E. Bradley—Unusual chrysophyceae studied in electron microscope. Plate 3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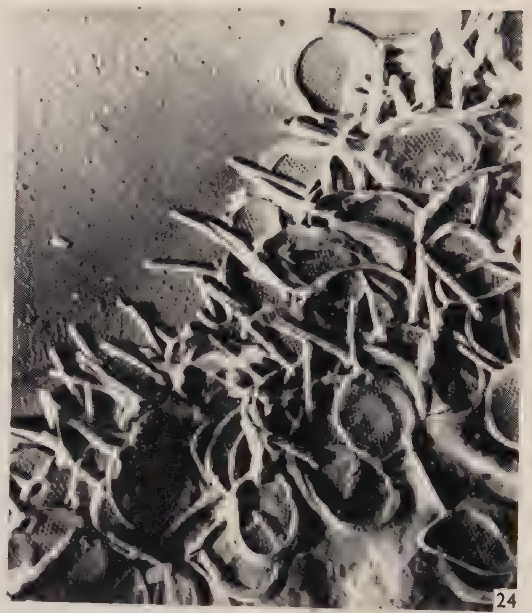
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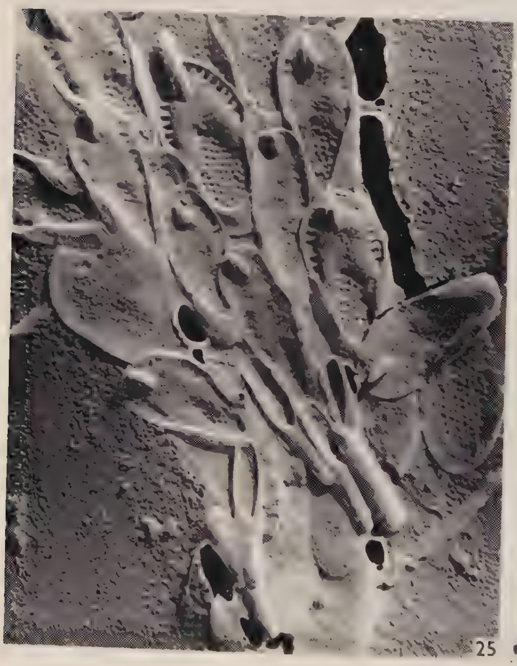
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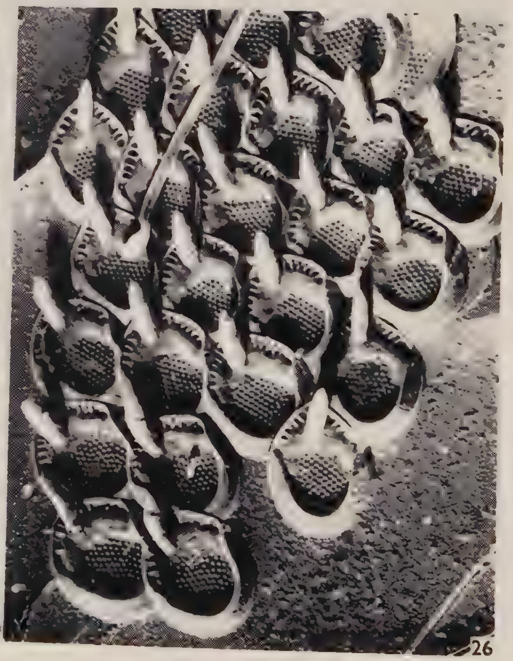
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K. Harris & D. E. Bradley—Unusual chrysophyceae studied in electron microscope. Plate 4

Fig. 10. *C. brevispina*, shadowed carbon replica of scales showing inner and outer surfaces; ×6000.

Fig. 11. *C. brevispina*, direct electron micrograph of scales; ×10,000.

Fig. 12. *C. brevispina*, shadowed carbon replica of goblet-shaped bristle base; ×5500.

Fig. 13. *C. brevispina*, fork-shaped bristle tip, direct electron micrograph; ×40,000.

PLATE 3

Fig. 14. *Synura sphagnicola*, direct electron micrograph of a scale with a spine; ×10,000.

Fig. 15. *S. sphagnicola*, shadowed carbon replica of inner surface of scale with spine, and also spoon-shaped scale; ×7000.

Fig. 16. *S. sphagnicola*, shadowed carbon replica of outer surfaces of scales without spines; ×8500.

Fig. 17. *S. sphagnicola*, direct electron micrograph of scale approaching spathulate shape; ×15,000.

Fig. 18. *S. sphagnicola*, shadowed carbon replica of a small circular scale; ×8000.

Figs. 19 and 20. *S. sphagnicola*, shadowed carbon replica of spathulate scales—right-hand scale in fig. 20 is inverted; ×9000.

Fig. 21. *S. sphagnicola*, direct electron micrograph of the tip of a spine similar to that in fig. 14; ×105,000.

Fig. 22. *S. sphagnicola*, direct electron micrograph of a small scale showing slits; ×120,000.

PLATE 4

Fig. 23. *Synura sphagnicola*, shadowed carbon replica of an array of scales surrounding protoplasm; ×4000.

Fig. 24. *S. sphagnicola*, shadowed carbon replica of another array; ×4000.

Fig. 25. *Synura echinulata*, shadowed carbon replica of the stalk; ×7000.

Fig. 26. *S. echinulata*, shadowed carbon replica of an array of scales (outer surface); ×5000.

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The Bacterial Flora of the Skin of North Sea Cod

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SUMMARY: Monthly bacterial counts over a 2-year period provided evidence of a seasonal variation in the numbers of bacteria on the skin of North Sea cod. The variations appeared to be connected with plankton outbursts. The temperature of the sea did not have any great effect, except that the proportion of the population capable of growth at 0° was highest when the sea temperature was at its lowest. The composition of the bacterial flora, determined by isolation of colonies from count plates after incubation at 20°, was: *Pseudomonas* 44 %, *Achromobacter* 32 %, *Corynebacterium* 9 %, *Flavobacterium* 6 %, *Vibrio* 6 %, *Micrococcus* 1 %, miscellaneous 2 %. When isolations were made after incubation at 0°, strains of the last four genera were less frequently encountered. Some evidence was also obtained of a seasonal variation in the qualitative composition of the flora, *Achromobacter* and luminous strains appearing more frequently in the winter while *Pseudomonas* strains increased in the summer. The flora of cod is similar to those of other species of North Sea fish examined by earlier workers; the species of the fish does not appear to play an important role in determining the composition of the skin flora.

Bacteria play a major role in the spoilage of fish as a foodstuff and the financial loss due to such spoilage has stimulated a considerable amount of investigation into the bacteria responsible; much of this work has been previously reviewed (Griffiths, 1937; Reay & Shewan, 1949; Shewan, 1949; Tarr, 1954). Besides investigations concerning spoilage, there have also been a few solely concerned with the bacterial flora of live or freshly caught sea fish. These have included the study of the floras of Canadian haddock (Reed & Spence, 1929), North Sea haddock (Stewart, 1932), Norwegian winter herring (Aschehoug & Vesterhus, 1943), Atlantic cod (Dyer, 1947) and North Sea skate and lemon sole (Liston, 1956, 1957). Except for Liston's work all these publications deal largely with the types of bacteria found on the fish and give very little information on variation in surface numbers or composition of the flora. That seasonal variations may occur was indicated by investigations on North Sea haddock by Reay & Shewan (1949), who suggested that these variations in fact reflected variations in the bacterial population of the environment, i.e. the sea water. Recent extensive investigations on North Sea skate and lemon sole also revealed seasonal variations (Liston, 1956). Liston found evidence that the increase in the surface flora of flat fish was related to seasonal outbursts of plankton in the surface layers of the sea. He postulated that these outbursts would be followed by a descent of detritus to the deeper layers of the sea, adding to and stimulating the local bacterial population of those layers.

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This in turn would cause increases in the surface bacterial populations of fish living at such depths.

Most workers agree that the surface bacterial flora of live marine fish is made up largely of Gram-negative rods (Shewan, 1949; Tarr, 1954; Liston, 1957). Nevertheless, there have been some reports that micrococci constitute a large proportion of the flora of marine fish (Wood, 1940; Dyer, 1947). Dyer worked on Atlantic cod and the large proportion of micrococci reported by her did not agree with findings on North Sea herrings, haddock and flat fish. This, together with the general lack of bacteriological data for North Sea cod, suggested the necessity for an investigation of the flora of this commercially important British fish. The present paper reports such an investigation, designed to provide information on the quantitative and qualitative aspects of the surface flora of cod sampled monthly during a period of 2 years.

METHODS

Treatment of fish. From January 1955 to January 1957, a batch of freshly trawled cod was examined each month. Each batch usually consisted of four fish taken from the same trawl. The trawling was performed in the North Sea, 10 miles east of Aberdeen, and, later, 30 miles south-east of that port. With only a minimum of handling the fish were placed in steam-sterilized metal boxes immediately after catching. The metal boxes, closed with sliding lids, were kept on ice during the trawler's return to harbour, and always arrived in the laboratory within 4 hr. of being caught.

Sampling and enumeration of microflora. On arrival, a skin sample, 7·5 cm^2. in area, with as little adhering flesh as possible, was taken aseptically from each fish and placed in a sterilized screw-capped 'universal' bottle containing 20 ml. sterile Ringer's solution and a little sterile sand. After shaking for 5 min. on an electrical shaker, duplicate roll tubes were prepared from serial dilutions, using the Astell apparatus. Three lots of roll tubes were set up from each sample and incubated at 37°, 20° and 0°. The medium used consisted of: Lab-Lemco, 5 g.; Difco peptone, 10 g.; Difco agar, 15 g.; aged sea water, 1000 ml. The pH value was adjusted to 7·4 and the medium sterilized at 15 lb./sq.in. for 15 min.

So that colonies could be subcultured easily, plate counts were carried out simultaneously on two samples from each batch, using the same medium and incubation temperatures as in the roll tube method. This was done during 1955–56 only. Periods of incubation, for both plates and roll tubes, were 3 days at 37°, 5 days at 20° and 25 days at 0°.

Analysis of microbial populations. This was done by direct and indirect methods. The luminous colonies appearing in the roll tubes were counted in a dark room. Bacteria resistant to penicillin were enumerated (during 1956–57 and at 20° only) by the roll-tube method, using the above medium containing 3 units penicillin/ml.

During 1955–56 randomly selected colonies were picked off the plates after incubation at 20° and 0°. Random selection was ensured by picking off all colonies occurring in one or more of the squares previously drawn on the plates. The plates with the largest number of discrete colonies were used. The colonies were transferred to sea-water agar plates, which were incubated at 20° for 7 days and then stored at 0° until required, usually after a month. Then they were transferred from the storage plates to tap-water nutrient broth, incubated at 20° for 5 days and checked for purity by plating on tap-water nutrient agar. Tap-water media were used in all subsequent investigations. A few strains did not grow in the tap-water broth when transferred from the sea-water agar but did so after cultivation for about a month in sea-water nutrient broth.

All the purified strains were examined for morphology, Gram-reaction, motility, gelatin liquefaction, action on litmus milk and reduction of nitrate (Griess-Ilosvay method). They were also tested for sensitivity to penicillin, chloramphenicol, streptomycin, oxytetracycline and a vibriostatic substance (2:4-diamino-6:7-di*iso*propylpteridine) as described by Shewan, Hodgkiss & Liston (1954); these tests were conducted at 20°. The antibiotic plates were read after 24 hr. and the gelatin and litmus milk tubes at intervals up to 28 days. Nitrate reduction was tested for after 7 days.

RESULTS

Quantitative

The average monthly counts at 20° and 0° are shown logarithmically in Fig. 1, which indicates that the counts at 20° and 0° varied between 10^3 and 10^6 organisms/cm^2. Since the counts at 37° on the same fish never rose above 5 % of those at 20°, they cannot be regarded as a true reflexion of the size of the bacterial population on fish. Fig. 1 also gives some indication of a regular seasonal variation. It seems that a general rise occurred in the counts at 20° in June and October 1955, and in July and November 1956. The counts at 0°, although showing similar variations, had the 1956 summer peak postponed until August. The counts at 0° were generally lower than those at 20°, though on a few occasions they approached or exceeded the latter (Fig. 2).

Qualitative

The monthly counts of luminous bacteria (1955–57) are shown in Table 1. This shows that the number of luminous bacteria increased in the winter months of each year; this increase did not coincide with the later increases in total count.

Table 2 shows the counts (during 1956–57) of bacteria resistant to penicillin, expressed logarithmically and as a percentage of the total 20° counts on the same fish. Although the number of penicillin-resistant bacteria showed a large rise in November, their proportionate representation in relation to the total flora was greatest in June and September.

Table 3 shows the distribution, according to genus, of the 1542 cultures isolated during 1955–56. It is obvious that the Gram-negative rod forms were the most common organisms encountered. Members of the less important

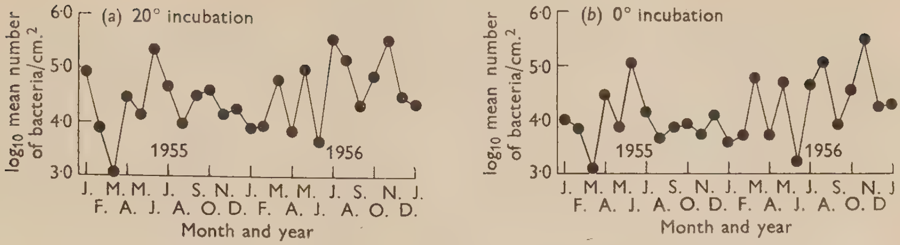

Fig. 1. Monthly bacterial counts (shown logarithmically) on the skin of cod; (*a*) 20° incubation; (*b*) 0° incubation.

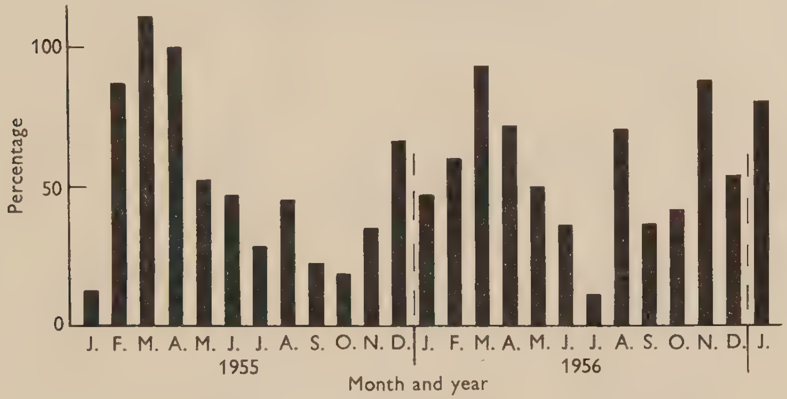

Fig. 2. The skin counts at 0° expressed as % of those at 20°.

Table 1. *Counts of luminous bacteria at 20° and 0°*

Month and year	No. of fish examined	Mean count/cm.² at 20°	Mean count/cm.² at 0°
Jan. 1955	2	13,230	160
Feb.	2	0	0
Mar.	4	0	0
Apr.	2	0	0
May	4	19	8
June	4	0	0
July	3	0	0
Aug.	4	0	0
Sept.	4	0	+*
Oct.	4	0	0
Nov.	4	0	0
Dec.	4	0	0
Jan. 1956	4	398	17
Feb.	4	1,636	270
Mar.	4	61	13
Apr.	4	21	5
May	4	0	0
June	4	+*	+
July	4	0	0
Aug.	4	27	+
Sept.	4	0	0
Oct.	4	+	0
Nov.	4	0	0
Dec.	4	+	0
Jan. 1957	4	133	0

* + indicates presence of luminous bacteria in very small numbers.

Table 2. *Counts of penicillin-resistant bacteria* (*at* 20°)

Month and year	No. of fish examined	log of mean count/cm.^{2}	As % of total 20° count
Feb. 1956	4	3·69	55·7
Mar.	4	4·27	29·7
Apr.	4	3·49	45·6
May	4	4·34	23·2
June	4	3·79	138·5
July	4	4·50	8·2
Aug.	4	4·49	20·1
Sept.	4	4·37	115·0
Oct.	4	4·79	84·0
Nov.	4	5·41	75·5
Dec.	4	4·09	39·8
Jan. 1957	4	4·20	70·0

Table 3. *Distribution, according to genus, of bacteria isolated from freshly caught cod*

	Number examined		
Bacterial genus	815 20° isolates (%)	727 0° isolates (%)	1542 All isolates (%)
Pseudomonas	44·0	51·5	47·5
Achromobacter	32·4	41·3	36·6
Corynebacterium	8·7	1·0	5·1
Flavobacterium	6·0	1·5	3·9
Vibrio	5·9	3·3	4·7
Micrococcus	1·1	0·7	0·9
Miscellaneous	1·9	0·7	1·3

genera, *Corynebacterium*, *Flavobacterium*, *Micrococcus* and *Vibrio*, were isolated more frequently from plates incubated at 20°. The distribution of the isolates in each of the 13 months is shown in Table 4. Members of the genus *Pseudomonas* were isolated less frequently in the winter months, while *Achromobacter* strains appeared to be more prevalent during this period. However, the fluctuations from month to month were so wide that it is difficult to distinguish any definite seasonal variation.

DISCUSSION

The quantitative results show that the numbers of bacteria on the skin of cod fall within the limits stated by Shewan (1949) for the bacterial load on freshly caught 'round' fish and are similar to those found on lemon sole and skate by Liston (1956). The variations reported above in the 20° counts on cod skin show similarities to those occurring on the skin of lemon sole (Liston, 1956), where the highest counts were reported to occur in the summer and early autumn, though on skate the highest skin counts occurred at the end of the year. Liston suggested that this might indicate a species effect; the difference between his findings on skate and the present findings on cod may be due to a similar effect.

During the two years of the present investigation, plankton outbursts occurred in the North Sea in the spring (February–April) and autumn (August–September) (Dr Fraser and Mr Lovegrove, personal communication). Devèze (1955) has shown that there is a period of about 3 or 4 weeks between an autumn plankton peak and the appearance of maximum numbers of bacteria in the sea water. The plankton outburst studied by Devèze occurred in the surface layers only (as with those in the North Sea) and it is quite possible that there would be a further delay before the deeper layers were affected. Because cod, like the flat fish examined by Liston, is also a bottom dweller, any increase in the bottom bacterial flora might be expected to have a similar effect to that with the flat fish. In 1955 there was a general rise in the skin flora of cod in June and, less pronounced, in October (Fig. 1), these periods being 1–3 months after the surface outbursts of plankton. In 1956 the higher counts were obtained in July and November and if, as seems possible, these peak loads were a delayed result of plankton outbursts, then the differences between 1956 and 1955 could be attributed to a slightly later plankton outburst or slower fall out of detritus in 1956. It has also been suggested that when a plankton outburst is at a maximum it may have an antagonistic effect on the bacterial population of the sea (Devèze, 1955). This could account for the decline in skin counts in March and April 1955 and September 1956, but the

Table 4. *Percentage distribution of bacteria isolated each month from cod skin during* 1955–56

			Composition of skin microflora													
	Colonies examined from plates incubated at		*Pseudomonas*		*Achromobacter*		*Corynebacterium*		*Flavobacterium*		*Vibrio*		*Micrococcus*		Miscellaneous	
			From plates incubated at													
	20°	0°	20°	0°	20°	0°	20°	0°	20°	0°	20°	0°	20°	0°	20°	0°
Month and year	No.		Colonies (%)													
Feb. 1955	43	40	44	35	44	60	0	5	12	0	0	0	0	0	0	0
Mar. 1955	98	40	40	35	41	55	6	5	10	0	0	0	2	5	1	0
Apr. 1955	76	38	55	76	9	21	25	0	6	0	3	0	0	3	2	0
May 1955	40	53	43	66	13	25	12	2	27	7	0	0	0	0	5	0
June 1955	69	45	68	66	16	34	9	0	3	0	1	0	0	0	3	0
July 1955	49	51	29	41	41	49	24	2	0	0	0	8	2	0	4	0
Aug. 1955	53	62	58	82	22	18	6	0	4	0	4	0	4	0	2	0
Sept. 1955	56	52	30	58	25	29	2	0	4	11	34	0	2	2	3	0
Oct. 1955	91	95	63	84	22	12	5	0	5	0	0	0	3	0	2	4
Nov. 1955	64	53	34	20	30	76	15	0	11	0	6	0	2	2	2	2
Dec. 1955	90	84	44	43	48	56	2	1	0	0	4	0	0	0	2	0
Jan. 1956	42	79	14	5	67	70	7	0	0	0	10	25	0	0	2	0
Feb. 1956	44	35	14	57	59	40	0	0	0	3	27	0	0	0	0	0

fluctuating results from February to June 1956 would seem to indicate that still other factors may have been operating.

While the temperature of the sea did not appear to influence the total number of bacteria, some effect of temperature can be seen from Fig. 2. The proportion of the population capable of growth at 0° reached peaks (Fig. 2) in the early spring months when sea temperatures were at their lowest, *c.* 5° (Dr Tait and Mr Craig, personal communication). It is as yet impossible to correlate this variation with the qualitative changes discussed below.

Evidence of qualitative variations is even more complicated than that for quantitative. Luminous bacteria were observed in only 12 of the 25 months of the investigation. When present they varied in numbers from 1 or 2 up to 13,200/cm.^{2}. The highest numbers occurred in the three winters of the investigation; this differs from the findings of Liston (1957). His results showed that these bacteria reached peak numbers on lemon sole and skate in the summer, and, on skate only, again at the end of the year. For both skate and lemon sole the numbers of luminous bacteria reported by Liston are larger and their appearance was more frequent than observed on the skin of cod during the present investigation; these differences in numbers and seasonal variations may be due to species effect. In general the present findings on cod are more akin to those for haddock, on which luminous bacteria were reported to be only infrequently observed (Stewart, 1932). The higher incidence of luminous bacteria on cod skin in the winter months might appear to be related to the low sea temperature at that time, but such a deduction would be inconsistent with the fact that larger luminous counts were obtained at 20° than at 0°.

During 1956 the number of bacteria resistant to penicillin followed the total count, but the proportion of these bacteria in the total count reached a pronounced peak at two periods in the year, namely June and September. This is similar to the results obtained from flat fish by Liston (1957), who used this method as a means of enumerating the pseudomonads present in the mixed flora of fish. In the present investigation it was hoped that these 'penicillin counts' for 1956–57 would give some confirmation of any seasonal fluctuations in the proportion of pseudomonads isolated during 1955–56. This was not possible because of the irregular variations in the proportions of pseudomonads that were isolated during that period.

As with the flora of the sea (ZoBell, 1946) and that of other species of North Sea fish (Liston, 1957) the flora of cod has been found to be composed mainly of Gram-negative rods. In contrast to Wood's (1940) and Dyer's (1947) findings only a small proportion of micrococci were isolated. The similar findings for several species of fish examined at Aberdeen make it unlikely that this difference is due to an effect of fish species. Liston (1957) has attributed such differences to environmental factors, other than species of fish, and this seems likely in view of the disparity between the present findings on North Sea cod and those of Dyer (1947) on Atlantic cod.

From the results of the isolation of cultures over a year, it seems possible that the higher proportions of *Achromobacter* strains isolated in the winter months are related to the lower sea temperatures around that period. However, other factors also appear to be operating since, in pure culture, *Achromobacter* and *Pseudomonas* strains from cod thrive equally well at 0°. Nevertheless, the results of the counts of luminous bacteria and those resistant to penicillin, together with the monthly distribution of the isolates, indicate the existence of seasonal variations in the composition of the bacterial flora of North Sea cod.

The present investigation also illustrates the importance of selecting a suitable incubation temperature for work on marine bacteriology. 37° was of no value at all, while 20° and 0° gave slightly different results on the qualitative side.

The author is indebted to Dr Fraser and Mr Lovegrove of the Marine Laboratory, Aberdeen, for information about outbursts of plankton and also to Dr Tait and Mr Craig, both of the same Station, for information about temperature fluctuations in the North Sea. The work described in this paper was carried out as part of the programme of the Food Investigation Organization, Department of Scientific and Industrial Research, and with the financial support of the South African Council for Scientific and Industrial Research.

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A Study of the Antigenic Specificity of Staphylococcal Coagulase in Relation to Bacteriophage Group

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SUMMARY: Rabbit antisera were prepared separately against coagulase from staphylococci of each of the three main bacteriophage groups. Neutralization tests showed that each of the three antisera caused considerable delay in the clotting of plasma by coagulase from the autogenous staphylococcus and other strains of the same phage group, but little or no delay in the action of coagulase from strains of other groups. Staphylococci of certain phage types yielded coagulase which was not neutralized by any of the three antisera. Amongst these were types 3A and 42E which are usually included in groups II and III, respectively, as well as unclassified types (e.g. 187). Antisera prepared against coagulase from types 3A and 42E caused significant neutralization of coagulase of the same type only.

Just over fifty years ago Loeb (1903–04) demonstrated the specific action of *Staphylococcus aureus* in causing clotting of goose plasma. This finding was confirmed by Much (1908), who emphasized the correlation between the clotting ability of staphylococci and pathogenicity. Subsequent workers have studied this correlation (Chapman, Berens, Peters & Curcio, 1934; Cruickshank, 1937) and the properties and mode of action of the clotting agent (Smith & Hale, 1944; Tager & Hales, 1948*a*, *b*; Duthie & Lorenz, 1952; Duthie, 1954). Contrary to the observations of earlier workers (Gross, 1931; Smith & Hale, 1944) it has recently been shown that staphylocoagulase is antigenic (Tager & Hales, 1948*b*; Rammelkamp, Hezebicks & Dingle, 1950; Duthie & Lorenz, 1952; Duthie, 1952). Rammelkamp *et al.* (1950) and Duthie (1952) found that coagulase derived from different staphylococci of human origin could be divided into three distinct antigenic types. Duthie also had a fourth antigenic type of coagulase from a strain of bovine origin. In later work Duthie (1954) showed that bound and free staphylocoagulase were distinct entities and immunologically unrelated.

The present study was concerned solely with free staphylocoagulase. It was begun in order to see whether there was any correlation between quantitative production of coagulase and virulence. In preliminary experiments to devise a simple quantitative test, however, significant differences in the optimum conditions of coagulase production and release were found with strains belonging to different bacteriophage groups. Artificial immunization of rabbits with coagulase preparations from staphylococcal strains of bacteriophage groups I and III were then prepared, and it was found that the two coagulases were of quite distinct antigenic types. A study of the antigenic specificity of coagulase in relation to bacteriophage type was, therefore, undertaken.

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METHODS

Organisms. All strains of staphylococci used in this study yielded free coagulase (Duthie, 1954) during growth in broth. Some of them had been recently isolated from patients and staff in St Thomas's Hospital, and some had been cultivated in the laboratory for many years. A number of other strains of representative bacteriophage types were kindly supplied by Dr R. E. O. Williams of the Central Public Health Laboratories at Colindale.

Selection of variants yielding large quantities of coagulase. For some purposes, especially for the production of neutralizing antisera, it was necessary to use staphylococcal strains which yielded large quantities of free coagulase. Duthie & Lorenz (1952) selected one such strain (Newman) by using fibrinogen plates; but strains of this sort seem to occur rarely in nature. Smith, Morrison & Lominski (1952) have, however, described 'fast clotting' variants which occurred in old cultures of staphylococci and which give rise to rough colonies on heated blood agar medium. Variants yielding large amounts of coagulase were therefore sought in cultures of a number of strains grown in different media. Six such variants isolated from three strains were sent for bacteriophage typing, together with their parent stock. In every case the phage patterns were similar.

Media. Three different media were used during this work. They were: peptone yeast-extract broth consisting of 1 % (w/v) peptone (Evans), 0·1 % (w/v) yeast extract (Difco), 0·5 % (w/v) NaCl, pH 7·2; bullock-heart infusion broth; fibrinogen agar which contained bovine fibrinogen (Armour fraction V), human plasma, trypsin inhibitor, nutrient broth and 1·5 % (w/v) agar (Difco; see Klemperer & Haughton, 1957). On the latter medium colonies of coagulase-positive staphylococci were surrounded by a halo of opacity whose diameter was usually found to be related to the concentration of coagulase formed when the organisms were grown in fluid culture.

Phosphate + citrate buffer. All dilutions of coagulase, plasma or fibrinogen were made in buffer which comprised 1 % (w/v) $NaH_2PO\ 2H_2O$ + 1 % trisodium citrate in distilled water. The pH value was adjusted to 7·0 with N-NaOH and the solution sterilized by autoclaving.

Production of free coagulase

Two methods were used for the production of high concentrations of coagulase.

Method 1. Selected variants were inoculated into 200 ml. amounts of peptone yeast-extract medium which were distributed in shallow layers in Roux bottles. These were incubated at 37° and sampled at intervals. When the samples were found to contain sufficient coagulase to give a clot in the standard test within 5 min. (after 1–3 days of incubation, depending on the strain), the organisms were removed by centrifugation and the coagulase in the supernatant fluid was concentrated by the cadmium sulphate method (see below).

Method 2. Another method for producing large yields of free coagulase has been developed by Dr E. S. Duthie (personal communication). The method depends upon the fact that coagulase is released by nearly all strains of *Staphylococcus aureus* in the presence of albumin (Davies, 1951; Duthie, 1954). For large-scale preparations, strains were inoculated into a number of 8 oz. medical flat bottles, each of which contained 5·0 ml. heart infusion broth. These bottles were mounted in a Kahn shaker and were continuously shaken for 18 hr. at 37°. After this, 45 ml. peptone yeast-extract medium to which had been added 0·15 % (w/v) bovine serum albumin (Armour fraction V), and which had been warmed to 37°, was added and the cultures again shaken for 2 hr. The cocci were then removed by centrifugation and the coagulase present in the supernatant fluid concentrated by the ammonium sulphate method (see below).

This method was also used on a smaller scale with 0·5 ml. heart infusion broth in test tubes 150 × 16 mm. After shaking for 18 hr., 4·5 ml. peptone yeast-extract medium with albumin were added.

Concentration of coagulase

Cadmium sulphate method. Coagulase produced by method 1 was concentrated by precipitation with 0·5 % (w/v) $CdSO_4$ at pH 5·8 in the cold (Duthie & Lorenz, 1952). After holding in the refrigerator overnight, the precipitate was collected, dissolved by the gentle addition of N-HCl to a final pH value of about 2·0, and then dialysed against running tap water in the refrigerator. When free from cadmium, the dialysed material was clarified by centrifugation and stored in the cool until required. This method of concentration was unsuitable when method 2 had been used for coagulase production, since under this condition the coagulase was not precipitated by cadmium sulphate. This was probably due to the presence of albumin.

Ammonium sulphate method. Coagulase produced by method 2 was concentrated by adding 2 vol. of saturated ammonium sulphate (Dr E. S. Duthie. personal communication). After storage overnight in the refrigerator the opalescent precipitate was collected, washed once in 60 % saturated $(NH_4)_2SO_4$ and taken up in distilled water.

Preparations of coagulase obtained by either of the above methods showed a 10- to 20-fold concentration.

Detection and estimation of free coagulase

The supernatant fluids from liquid cultures or concentrated preparations obtained as described above were tested for their ability to clot fresh citrated human plasma. With the supernatant fluids, further elaboration of coagulase by remaining bacteria was prevented by adding sodium thiomersalate to give a final concentration of 1/10,000 (Davies, 1951). For the test, 0·2 ml. volumes of suitable dilutions were mixed with an equal volume of clotting system in a 75 × 8 mm. test tube and incubated at 37° in a water bath. The formation of a clot indicated free coagulase. The test was made quantitative by measuring the time taken for a clot to develop. Dilutions which gave clots in from 5 to 60 min. gave the most accurate results. No attempt was made to convert the readings into 'units' of activity; they have been recorded directly as clotting times in minutes.

Clotting system. Fresh citrated human plasma, always taken from the same individual, was made up to 20 % (v/v) in buffer, and bovine serum albumin (Armour fraction V) was added to give a final concentration of 3 % (w/v).

Preparation of antisera

Strains with high coagulase activity and representative of each of the three main bacteriophage groups were first selected as follows: strain M, phage-type 52/80+ (Group I); strain W120, phage-type 3B/3C/55+ (group II); and strain Newman, phage type 7/47/53/54/73/75/77 (group III). Later one strain each of phage types 3A and 42E were also used. Antigens for injection were prepared by adding 0·4 g. potassium aluminium sulphate to 15 ml. of coagulase preparation and adjusting the pH value to 6·8 with N-NaOH. Preparations of coagulase concentrated by either method were used, but those prepared by ammonium sulphate precipitation were found to have a high toxicity which could not be attributed to α-toxin. The coagulase activity of different preparations was very variable and was not standardized.

Antisera were prepared by injecting young adult rabbits with 0·5–1·0 ml. doses of alum-precipitated coagulase antigen once weekly for 4 or more weeks. Ten days after the last injection, the rabbits were bled and their sera separated. Sera were inactivated at 56° for 30 min. before use.

Coagulase neutralization test

Coagulases, either in the form of concentrated preparations or supernatant fluids of cultures after centrifugation, were used. After testing their activity the coagulase preparations were diluted to give a clot after incubation for 10 min. but not after 5 min. These dilutions were mixed with equal volumes (0·1 ml.) of serum, usually diluted 1/2, and left at room temperature for 1 hr. The residual activity was then measured by adding 0·2 ml. clotting system incubating at 37° and noting the clotting time.

Haemagglutination

Coagulase was adsorbed on to tanned erythrocytes (Boyden, 1951). Both human (group O) and sheep cells were used. After washing three times in the phosphate citrate buffer, 1 ml. amounts of packed cells were suspended in 9 ml. buffer containing 1/20,000 tannic acid (British Drug Houses Ltd.) and left at room temperature for 10 min. The tanned cells were then separated by centrifugation and washed once in buffer. Nine ml. of coagulase preparation at different concentrations was left in contact with 1 ml. of packed tanned cells for 10 min. The cells were then washed once and resuspended in buffer containing 0·4 % (v/v) normal rabbit serum. For haemagglutination tests 1 % (v/v) suspensions of cells were added to equal volumes of antiserum dilutions, and the results read after sedimentation.

RESULTS

Preliminary observations

Many preliminary experiments were made to determine the conditions under which different strains of *Staphylococcus aureus* gave high yields of free coagulase in liquid cultures. In peptone yeast-extract medium a significant difference was found between the behaviour of strains of bacteriophage groups I and III. The difference was particularly marked in relation to the effect of adding sera or albumin from various animals. A typical experiment is shown in Table 1, from which it will be seen that strains of group I produced little or no free coagulase except when grown in the presence of human serum, whereas strains of group III yielded moderate or large amounts of free coagulase under most conditions. It is interesting, however, that guinea-pig and cat sera appeared to inhibit either the production or activity of coagulase.

Table 1. *Effect of sera of various animals on the production of free coagulase by staphylococci*

22 hr. shaken cultures in peptone yeast-extract medium

	Bacteriophage group of staphylococci					
	I			III		
	Strain of staphylococcus					
	M	SB 25	SB 26	SB 21	N	Woods
Medium	Relative amounts of coagulase					
Broth alone (v/v)	–	–	–	+++	++++	++
Broth + 1 % (v/v) bovine albumin	–	–	–	++++	++++	++
Broth + 10 % (v/v) human serum	+++	+++	+++	++++	+++	+++
Broth + 10 % (v/v) horse serum	+	+	+	+++	++++	+
Broth + 10 % (v/v) rabbit serum	–	–	–	+++	++++	+++
Broth + 10 % (v/v) fowl serum	–	–	–	+	++++	–
Broth + 10 % (v/v) guinea-pig serum	—	—	—	–	++	–
Broth + 10 % (v/v) cat serum	–	–	–	–	–	–

Clotting times of supernatant fluids + thiomersalate recorded as follows: ++++ = < 10 min.; +++ = 10–60 min.; ++ = 360 min.; + = clot overnight; – = not clot.

Production and release of free coagulase

The above results are recorded because they first called to our notice a difference between staphylococcal strains of different phage groups. In all further work the two methods described above for production of free coagulase were used. Consistently high yields were obtained by method 2 with nearly all strains of *Staphylococcus aureus* tested, and this method was, therefore, used in all coagulase neutralization tests. Method 1 had the advantage that no albumin was added, and was therefore preferred for preparation of antisera, but good yields of free coagulase were only obtained by this method with the freak strain, Newman, or with 'fast-clotting' variants selected from other strains.

Selection of 'fast-clotting' variants

Twenty-four strains of *Staphylococcus aureus* were examined for the occurrence of variants giving high and low yields of free coagulase. Broth cultures were plated out on fibrinogen plates and the area of opacity round individual colonies noted. It was frequently found that colonies from a single strain showed gross variation in this respect. Colonies surrounded by large and small haloes were selected from each strain and tested for production of free coagulase in peptone-yeast broth, without the addition of serum or albumin. By this technique cultures showing gross variations in coagulase activity were isolated from 18 of the 24 strains. Typical results with 6 strains are shown in Table 2. The colonies of the 'fast-clotting' variants isolated in this way were often small and rough in appearance, as described by Smith *et al.* (1952). The surrounding area of opacity was always large, although a direct quantitative relationship between this and production of free coagulase in broth has not been demonstrated. Typical appearances are seen in Pl. 1.

Table 2. *Production of free coagulase in peptone-yeast broth by selected colonies from 6 strains of* Staphylococcus aureus

	Colony *a*	Colony *b*
	Clotting time (min.)	
Strain		
M	<5	45
26	<5	45
W120	2	360
PR	2	60
LKS	10	360
LAR	<1	360

Evaluation of coagulase test

Relationship of coagulase concentration to time of clot. Tests were carried out in replicate with different dilutions of a preparation of coagulase, and the tubes were examined for clot formation every 2·5 min. The following results were obtained.

Dilutions ...	1/1	1/2	1/4	1/8
No. of replicates	10	10	20	20
Mean clotting time (min.)	6·75	11·25	29·5	55·5
Standard deviation	1·2	0·26	2·04	5·5

In a further experiment closer dilutions were used and the results are shown in Fig. 1. It will be seen that the relationship is linear over a period from 5 min. to 1 hr.

Factors which affect the tests. 20 % (v/v) fresh citrated human plasma from one individual was used for all tests. In early work it was found that the rate of clot formation was affected by the presence of albumin. Addition of bovine serum albumin to give a final concentration of 3 % (w/v) caused a two- to sixfold decrease in clotting time with all coagulase preparations tested. This was, therefore, incorporated in the clotting system (see Methods). Later, when testing the effect of rabbit antisera, it was found that the presence of normal rabbit serum accelerated clot formation to a variable extent. This effect was most marked when the coagulase preparation and rabbit serum were left in contact with one another for a period before plasma was added, and was not altered by inactivating the serum at 56° for 30 min. For this reason, in coagulase neutralization tests, controls were left in contact with a volume of normal rabbit serum equivalent to that of the antiserum used in the tests.

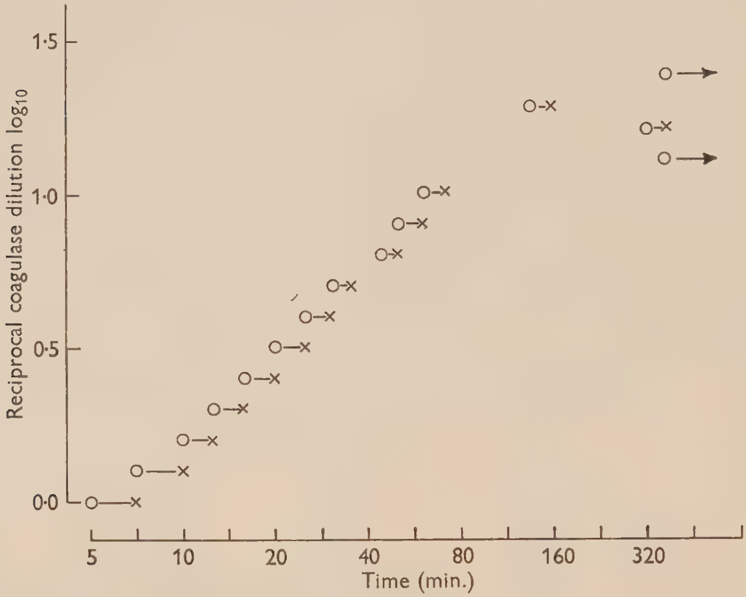

Fig. 1. Relationship between clotting time and dilution of coagulase. Both axes are plotted on logarithmic scales. O, last negative reading; ×, first positive reading.

Coagulase neutralization with rabbit antisera prepared against coagulase from staphylococcal strains of bacteriophage groups I, II and III

Activity of antisera. At first antisera were prepared against coagulase from one strain of staphylococcus from each of the three major bacteriophage groups. The neutralizing activity of each antiserum was tested after four or five injections of antigen. In most cases it was found that the antiserum possessed moderate or good neutralizing power against the homologous coagulase, but in some cases it was necessary to give a further course of injections. The factors which affected antibody response were not investigated, but in all cases the antiserum finally used possessed good neutralizing ability against the homologous coagulase. Attention was, however, paid to the effect on neutralization of altering the proportions of coagulase and antibody present. A chessboard type of experiment was set up in which falling dilutions of antiserum (1/1–1/100) were each tested against falling dilutions of coagulase; the results are given in Table 3. Two practical points emerge. First, the antiserum was of low titre in the sense that however dilute the coagulase only low dilutions of serum had an effect; yet the antiserum was of high activity in the sense that these dilutions enormously prolonged the clotting time of all dilutions of coagulase. Secondly, small alterations in the dilution of coagulase had a dramatic effect on the degree of neutralization. In neutralization tests, therefore, antisera were always used at low dilution (1/2 or 1/4) and the coagulase preparations were carefully standardized to give a clot in 5–10 min., which corresponds to the dilution 0·6 log$_{10}$ in Table 3.

Table 3. *Coagulase neutralization; effect of varying proportions of coagulase and antiserum*

The figures in the main part of the table represent the factors by which clotting times were delayed; — = delayed beyond the limits of experiment.

	Dilution of coagulase (log$_{10}$)				
	0	0·3	0·6	0·9	1·2
Dilution of antiserum (log$_{10}$)	Relative delays in clotting time				
0	3000	—	—	—	—
0·2	56	—	—	—	—
0·4	10	—	—	—	—
0·6	2·8	500	—	—	—
0·8	1	14	144	—	—
1·0	1	2·8	8	78	—
1·2	1	1	2·5	7·5	62
1·4	1	1	1·25	2	2·4
1·6	1	1	1·25	2	1·4
1·8	1	1	1	1·5	1·4
2·0	1	1	1	1·5	1

Specificity of antisera. The neutralizing effect of each of these sera was then tested against each of the three preparations of coagulase; the results are shown in Table 4. It is clear that each antiserum has a dramatic effect on the

Table 4. *Coagulase neutralization by rabbit antisera*

Clotting times were determined after contact for 1 hr. at room temperature with rabbit sera diluted 1/2.

	Normal rabbit serum	Rabbit antisera		
		I (M)	II (W120)	III (N)
Coagulase preparation	Clotting time (min.)			
I (M)	10	270	10	10
II (W120)	10	10	180	10
III (N)	15	10	10	>1440

homologous coagulase and none whatever on the others. We therefore decided to test coagulase from a number of strains of *Staphylococcus aureus* of known bacteriophage type.

Through the kindness of Dr R. E. O. Williams, of the Central Public Health Laboratory, we obtained a number of strains of commonly occurring phage types. Coagulase from each of these was tested against each antiserum. Clear evidence of neutralization by one only of the three antisera was noted with 42 of 49 staphylococcal strains tested. When the results were analysed in relation to bacteriophage group the correlation was good. It will be seen from Table 5 that there are few discrepancies and all of them are interesting.

Table 5. *Neutralization by rabbit antiserum of coagulase from* 49 *strains of* Staphylococcus aureus *of different bacteriophage groups*

Phage group	No. of strains tested	Average factor by which clotting time is increased			Remarks
		Antiserum I	Antiserum II	Antiserum III	
Group I	14	9·3 (>3–18)*	1·1 (0·75–2·0)	1·0 (0·6–1·5)	—
Group I	1	1·0	1·5	1·0	Bovine strain
Group II	8	1·4 (1·0–1·5)	5·0 (>2·5–8·0)	1·1 (1·0–2·0)	—
Group II	2	1·3 (1·3 and 1·3)	1·0 (1·0 and 1·0)	1·0 (1·0 and 1·0)	Both type 3A
Group III	19	1·1 (0·1–1·5)	1·0 (0·5–2·0)	14·0 (>6–36)	—
Group III	2	1·8 (1·5 and 2·0)	1·4 (1·3 and 1·5)	1·2 (1·0 and 1·3)	Both type 42E
Miscellaneous	2	1·4 (1·3 and 1·5)	1·3 (1·0 and 1·5)	1·0 (1·0 and 1·0)	Both type 187
Non-typable	1	>4·0	0·6	0·6	—

* Range is given in parentheses.

The one anomalous strain of phage group I was atypical in that it was of bovine origin. In group II both outstanding strains were of phage type 3A and they were the only strains of this phage type. A similar anomaly was noted in group III where the two exceptional strains were alone in being of phage type 42E. Strains of type 187 do not fall into any of the three major phage groups and our results confirm this. The non-typable strain would appear to have originated from a strain of phage group I. Two strains sensitive to phages of both groups I and III were also tested. The coagulase from one of these was specifically neutralized by antiserum I whilst that of the other was not affected by any of the antisera.

A further 30 strains of *Staphylococcus aureus* isolated in an investigation by Hare & Ridley (1957, unpublished) were typed by bacteriophage at the Central Public Health Laboratory, and tested by the coagulase neutralization method. Twenty-one of the strains fell clearly into one or other of the three main phage groups and with 20 of these the coagulase neturalization test gave good agreement. The remaining strain was classified as phage type 54/77+ but only reacted to concentrated phage preparations; by the coagulase neturalization test it appeared unequivocally to be group I. Seven of the 30 strains reacted to phages of both groups I and III. Of the coagulases from these, 3 were neutralized by group I antiserum, 2 by group III antiserum, 1 was inhibited slightly by all three antisera and 1 was not affected by any of the antisera. Two strains of miscellaneous phage type (69 and 42D) also failed to react with any of the antisera.

Coagulase neutralization with further rabbit antisera

In the light of these findings antisera were prepared against coagulases from one strain of each of the types 3A and 42E. Neutralization tests of coagulases from 26 representative strains were done with these and the 3 original antisera. The results, shown in Table 6, confirm the finding that coagulase from strains of type 3A differs significantly from that of other group II strains, and similarly that coagulase from type 42E is distinct from that of other group II strains. It will be seen that the 5 antisera differed considerably in the degree of activity against coagulase of the homologous group. No attempt has so far been made to standardize the antisera.

Table 6. *Neutralization by rabbit antiserum of coagulase from 26 strains of* Staphylococcus aureus *of different bacteriophage patterns*

Phage pattern	No. of strains tested	Average factor by which clotting time is increased				
		Antiserum I	Antiserum II	Antiserum III	Antiserum 3A	Antiserum 42E
Group I	6	10·7 (6·0–18·0)*	1·2 (1·0–2·0)	1·0 (0·5–1·5)	3·3 (1·5–5·5)	1·6 (1·0–2·5)
Group II	8	1·3 (1·0–2·5)	> 5·3 (> 2·5–> 8·0)	1·2 (1·0–2·3)	1·0 (1·0–1·0)	1·1 (1·0–2·5)
Group III	8	1·2 (1·0–1·5)	1·1 (1·0–2·0)	> 15·5 (> 6·0–36·0)	1·5 (1·0–2·5)	1·6 (1·0–2·5)
Type 3A	2	1·0 (1·0 and 1·0)	1·0 (1·0 and 1·0)	1·3 (1·3 and 1·3)	> 12 (> 12 and > 24)	1·0 (1·0 and 1·0)
Type 42E	2	1·4 (1·3 and 1·5)	1·4 (1·3 and 1·5)	1·5 (1·0 and 2·0)	2·5 (2·0 and 3·0)	6·5 (5·0 and 8·0)

* Range is given in parentheses.

Anomalous strains

Two strains of type 42D gave unexpected results. The coagulase of one (W) showed significant inhibition with group III antiserum, whereas that from the other (T) was unaffected. An antiserum was prepared against W coagulase. This caused a <12-fold delay in the action of the autogenous coagulase and a 4 to 16-fold delay in that of all 8 strains of staphylococci of bacteriophage group III, but failed to inhibit coagulase from the other 42D strain. When tested with additional phages the two strains differed in that one (W) was strongly lysed by phage 52B, whereas the other (T) was not.

Coagulase from one strain of type 53 was only weakly inhibited by group III antiserum. An autogenous antiserum delayed its action by a factor of 10 and that of 15 strains of group III by a factor of 1·6–5·3 (average 3·7). In fact this antiserum affected the coagulase of some strains from all phage groups to a similar extent.

Coagulase neutralization by human sera

The coagulase neutralizing activity of sera from 29 human adults was tested against coagulase from 17 strains of *Staphylococcus aureus* representative of the three main phage groups and types 3A, 42E and 187; the results are shown in Table 7. It will be seen that nearly all sera contained antibodies to group I coagulase, more than half to groups III and II, and about a third to each of the remainder. Only 2 sera had no antibodies to any coagulase. It is significant, in view of our findings with rabbit antisera, that antibodies to coagulase of type 3A did not necessarily go with those to group II; similarly, with type 42E and group III. One serum inhibited the coagulase from only 5 out of 6 group I strains and another only 1 of 2 strains of type 187. In all other instances consistent results were obtained.

Table 7. *Pattern of staphylococcal coagulase-neutralizing antibodies in 29 human sera*

No. of sera	I	II	3A	III	42E	187
4	+	+	−	−	−	−
3	+	−	−	−	−	−
2	+	+	−	+	−	+
2	+	+	−	+	−	−
2	+	+	+	+	+	+
2	+	+	+	+	+	−
2	+	−	−	+	+	−
2	−	−	−	−	−	−
2	+	+	+	+	−	+
1	+	−	−	−	−	+
1	+	−	−	+	+	+
1	+	−	+	+	+	+
1	+	−	+	+	+	−
1	−	−	−	+	−	−
1	+	−	−	−	+	+
1	+	+	−	+	+	+
1	+	−	+	−	−	−
Total 29	26	15	9	17	11	11

Haemagglutination

Because of the specificity of our results with the coagulase neutralization test, it was thought desirable to see whether similar specificity could be demonstrated by another technique. Coagulase was found to be readily adsorbed on to tanned, but not untanned, human and sheep red blood cells. Thus when preparations of coagulase which gave clotting in 10 min. were left in contact with one-tenth volume of tanned erythrocytes for 10 min., 25–50 % of the coagulase was adsorbed. Cells coated in this way by coagulase preparations of each of the three main groups of staphylococci were agglutinated by all three antisera to a similar degree, but not by normal rabbit serum. Since this demonstrated the impurity of the coagulase preparations, serial adsorptions with varying proportions of red cells were carried out. This procedure increased the specificity of haemagglutination in some experiments, but so far we have failed to standardize the method,

DISCUSSION

The results presented here show that there is a close relationship between the bacteriophage group of staphylococcal strains and the antigenic specificity of the free coagulase produced by the corresponding strains. Thus antiserum prepared against coagulase from staphylococci of one bacteriophage group neutralizes coagulase from most strains of the same group, but not coagulase from strains of other groups. The coagulase from staphylococci of phage type 3A appears distinct from that of other group II strains. Similarly, that from type 42E differs antigenically from that of other group III strains. Of 80 strains tested, apart from 7 of unclassified or mixed phage patterns, only 2 did not fall into one or other of these 5 coagulase groups.

In 1922 Julianelle in America and Hine in Great Britain divided pathogenic staphylococci into three serological types by means of agglutinating antisera. Subsequent workers have confirmed by more refined techniques the three main groups, and demonstrated other serological types or sub-types (Cowan, 1938; 1939; Christie & Keogh, 1940). Hobbs (1948) made an extensive study of the serological classification of staphylococci. By means of agglutination tests with absorbed sera, she recognized 7 specific serological types and a further 8 types with less specific reactions.

Staphylococci have also been classified by their susceptibility to bacteriophages. Fisk (1942*a*, *b*) showed that phages carried by many strains of *Staphylococcus aureus* lysed some but not all other strains. Wilson & Atkinson (1945), who used bacteria-free phage filtrates, defined 21 different phage types among pathogenic staphylococci. Williams, Rippon & Dowsett (1953), with 24 different phage filtrates, found that 1394 strains of *Staphylococcus aureus* fell into three broad groups. These corresponded to the three serological groups recognized by Cowan (1939). Hobbs (1948) showed a relationship between serological type and bacteriophage pattern and reading this paper in the light of the phage groups defined by Williams *et al.* (1953) and of our own results, it seems of interest to note that Hobbs found that strains of phage type 3A and 42E differed serologically from most strains lysed by other phages of group II or group III respectively.

The capacity of coagulase to produce antibodies has only recently been appreciated. Lominski & Roberts (1946) demonstrated a specific coagulase inhibitor in human sera, which was associated with the globulin fraction. Tager & Hales (1948*b*) were the first to record the experimental production of antibodies to coagulase. Rammelkamp *et al.* (1950) distinguished three antigenic types of coagulase by neutralization tests with sera from 148 medical students and 3 immunized monkeys. Duthie & Lorenz (1952) and Duthie (1952) by using antisera prepared in rabbits and 25 human sera were able to define 4 antigenic types of coagulase, one of which was derived from a bovine strain. None of these workers related coagulase type to bacteriophage type.

The close relationship which we have found between antigenic type of coagulase and bacteriophage type suggests either some direct association between coagulase and phage, or a difference in the genetic constitution of staphylococci of different phage groups. Possible direct relationships are that coagulase acts as a receptor for phage or is concerned in events after absorption. Preliminary experiments to demonstrate such a relationship have been inconclusive.

A number of factors point to a difference in the genetic constitution of staphylococci of each of the three main phage groups. Besides the serological distinction we found differences in production and release of coagulase. Other workers have drawn attention to the association of certain phage groups of staphylococci with particular types of infection (cf. Anderson & Williams, 1956). Moreover, there is evidence to suggest that staphylococci of phage group III yield antibiotic-resistant variants more readily than do staphylococci of other groups (Barber & Whitehead 1949; Rountree & Thomson, 1949; Barber & Burston, 1955).

All investigators who have used agglutination or precipitation techniques have found cross-reactions so common that unabsorbed sera had little or no distinguishing value. It is remarkable that with coagulase neutralization, probably because of the crudity of the test and the fact that a single antigen is concerned, cross-reactions are relatively unimportant. This means that it has advantages over other methods of serological classification, and although it distinguishes fewer types than the bacteriophage method, it might be a useful procedure in small laboratories.

The presence of coagulase-neutralizing antibodies in human sera have been shown to be of common occurrence (Lominski & Roberts, 1946; Rammelkamp *et al.* 1950; Duthie 1952). We confirm the findings of Rammelkamp *et al.* (1950) and Duthie (1952) that an individual may have antibodies to one type of coagulase but not to another. Thus the incidence of neutralizing antibodies in human sera varies with the coagulase used for the test. Of the 29 individuals studied by us 26 had antibodies to coagulase of group I, 17 to that of group III and 15 to that of group II. Antibodies to coagulases from staphylococci of types 3A, 42E and 187 were less frequent. These results reflect the ubiquity of staphylococcal infection; and the high incidence of antibodies to group I coagulase parallels the frequency of small circumscribed infections, e.g. boils, due to staphylococci of this group.

It is a pleasure to thank Dr E. S. Duthie for valuable information, Dr R. E. O. Williams and Dr Joan Rippon for supplying strains and for the bacteriophage typing, and Mr A. L. Wooding for the photographs.

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EXPLANATION OF PLATE

Fig. 1. Fibrinogen plate inoculated with cultures from 2 strains of *Staphylococcus aureus*, A and B. Growths on the left come from parents cultures and those on the right from 'fast-clotting' variants.

Fig. 2. A single strain (W120) of *Staphylococcus aureus* plated out on fibrinogen agar. Three types of colony will be seen: (*a*) large colonies with no halos, (*b*) large colonies with moderate halos, (*c*) small colonies with large halos (fast-clotting variants).

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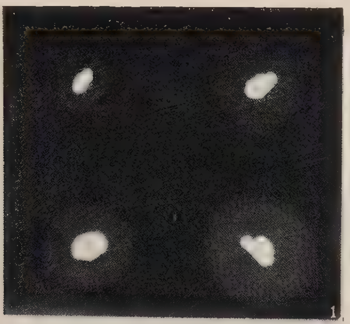

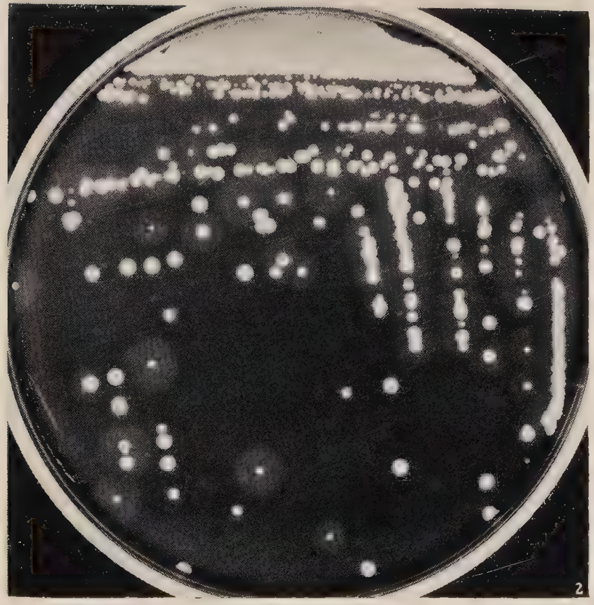

M. Barber & P. Wildy—Staphylococcal coagulase. Plate 1

(*Facing p.* 106)

SAMEJIMA, H. & MYERS, J. (1958). *J. gen. Microbiol.* 18, 107–117

On the Heterotrophic Growth of *Chlorella pyrenoidosa*

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SUMMARY: Of a large number of possible carbon sources only glucose, galactose, and acetate supported continued growth of *Chlorella pyrenoidosa* in darkness. Cell synthesis in darkness with nitrate as the nitrogen source proceeded with a carbon assimilation of 45 % for glucose, 37 % for galactose, and 26 % for acetate. The energy efficiency (Rubner coefficient) of cell synthesis from glucose was affected by the nitrogen source and has a maximum value of 58 % with ammonia.

The spectrum of nutritional characteristics found among the algae ranges from obligate autotrophy to obligate heterotrophy. A number of species in the genera *Chlorella* and *Scenedesmus* lie near the midpoint of the spectrum in the sense that they shift rapidly and reversibly between growth in darkness on organic substrates and growth in light on carbon dioxide. Ability to utilize organic substrates appears subject to wide variation between species and strains (Algeus, 1946; Bristol Roach, 1927; Fogg, 1953; Killam & Myers, 1956; Neish, 1951). For example, the Columbia strain of *Chlorella vulgaris* utilizes glucose, fructose, galactose and four β-glucosides for growth in darkness (Neish, 1951). In contrast, it has seemed surprising from the more casual observations of this laboratory that the Emerson strain of *C. pyrenoidosa* must be far more limited in its ability to utilize organic carbon sources. The present work examines the utilization of carbon sources by *C. pyrenoidosa* with some observations on two other species. Quantitative data will be presented on growth rates, yields, and efficiencies of substrate utilization for cell synthesis.

METHODS

The algal species studied included *Chlorella pyrenoidosa* (Emerson strain), *C. ellipsoidea* (from Dr H. Tamiya) and *Scenedesmus* sp. D3 (from Dr H. Gaffron).

The basal medium was a Knop's solution of the following composition (g./l.): 1·25, KNO_3; 1·25, KH_2PO_4; 2·5, $MgSO_4.7H_2O$; 0·30, Na citrate; 0·004, $Fe_2(SO_4)_3$; 0·0029, H_3BO_3; 0·0018, $MnCl_2.4H_2O$; 0·00022, $ZnSO_4.7H_2O$; 0·000080, $CuSO_4.5H_2O$; 0·000018, MoO_3. The pH value was adjusted with KOH to 5·6–5·8. Organic substrates at twice final concentration were filter-sterilized or autoclaved separately and mixed with an equal volume of autoclaved basal medium of double strength.

Growth rates were measured photometrically in test-tube cultures containing 15 ml. medium aerated with 5 % (v/v) carbon dioxide in air and immersed in water baths thermostated at 25 ± 0·1°. The cultures were enclosed in a dark chamber or illuminated by fluorescent lamps from both sides of the bath.

Each culture was inoculated with 1·0 ml. of an algal suspension grown photosynthetically in basal medium. For *Chlorella ellipsoidea* and *C. pyrenoidosa* inocula were taken from a continuous culture apparatus. At intervals the optical density was observed in an Evelyn colorimeter with a 600 mμ. filter. The logarithm of optical density plotted against time gives a curve (cf. Fig. 1), the slope of which is the specific growth rate. Values of the specific growth rate k will be reported in the convenient dimensions of $\log_{10}$ units/day as for the equation $\log_{10} N/N_0 = kt$.

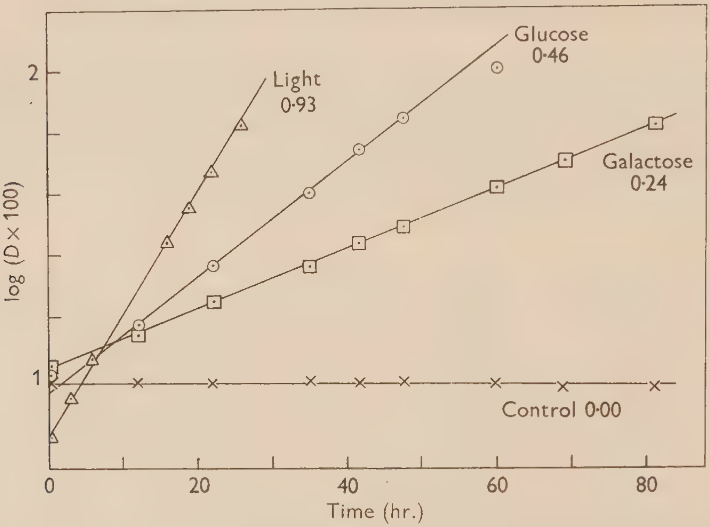

Fig. 1. Growth curves in terms of optical density (D). *Chlorella pyrenoidosa* at 25° in basal medium aerated with 5 % (v/v) CO_2 in air, in light, in darkness (control), and in darkness with addition of 1 % of the substrate indicated. The figures underneath the curves represent values of the specific growth rates.

Growth yield was determined in cultures grown in 200 ml. test tubes in darkness at 25° and aerated with 5 % (v/v) carbon dioxide in air. Samples of medium containing known amounts of carbon source were distributed to each of a series of tubes. Organisms for inoculation were washed twice in water with aseptic precautions and 1·0 ml. of suspension delivered to each tube. The cultures were harvested at intervals by centrifugation, and the organisms washed thrice in water and dried at 100–110° to constant weight in tared crucibles. One of the growth curves thus obtained is shown in Fig. 2. Yield of organism was obtained by subtraction of initial weight of organism (inoculum) from the maximum weight of organism developed. For three experiments cells from cultures at maximum yield were prepared by lyophilization for elementary analysis. Lyophilization was chosen since cells dried at 100–110° from a suspension in water gave erratic values for ash content. The elementary analyses reported were obtained from the Biochemical Institute, University of Texas.

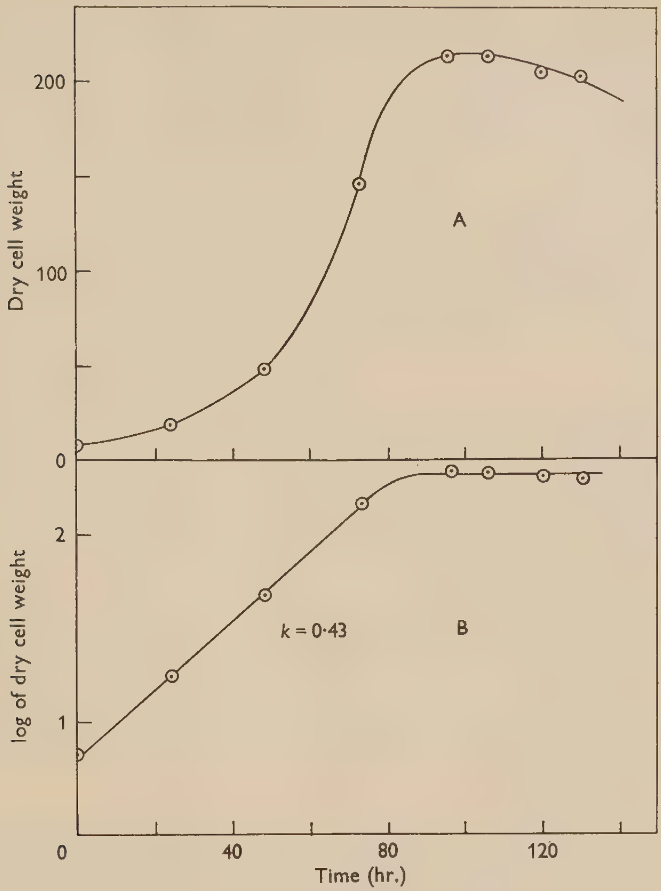

Fig. 2. A growth curve typical of cell yield experiments (A) plotted also on a logarithmic basis (B). The experiment shown provided the eighth horizontal column of data of Table 3.

RESULTS

Growth-rate studies

Sugars and sugar-alcohols in darkness. The following were tested at a concentration of 1 % (w/v): arabinose, xylose, ribose, glucose, mannose, galactose, fructose, maltose, sucrose, lactose, cellobiose, glycerol, mannitol, *i*-inositol. Of these only glucose and galactose clearly supported growth of the two Chlorellas. Good growth of *Scenedesmus* sp. D3 was obtained only on glucose, although slow ($k < 0{\cdot}06$), but continued growth was supported by galactose, mannose, fructose, maltose, sucrose, or lactose. For the Chlorellas xylose, arabinose, fructose, and maltose supported a small amount of growth which persisted only during the first 30–70 hr. Two explanations are possible: (1) a small amount of assimilable carbon source occurs in these sugars as an impurity, or (2) these sugars are utilized as long as there exist in the cells some substances required for priming reactions.

Sugars at different light intensities. It has been observed that very low illumination markedly accelerates growth of *Chlorella vulgaris* (Emerson strain) on glucose (Killam & Myers, 1956). Results of a search for the same phenomenon with the present Chlorellas on five sugars is presented in Table 2. Two different light intensities were obtained by filtering the fluorescent illumination through layers of paper. The data of Table 2 may be examined

Table 1. *Growth rates of* Chlorella pyrenoidosa, C. ellipsoidea *and* Scenedesmus *sp. D3 on various carbon sources*

Growth at 25° in basal medium of initial pH 5·6–5·8 aerated with 5 % (v/v) Co$_2$ in air; values of specific growth rate are expressed in $\log_{10}$ unit/day.

	C. pyrenoidosa	*C. ellipsoidea*	*Scenedesmus* D3
	Specific growth rate ($\log_{10}$ unit/day)		
In darkness			
Single substrate (%, w/v)			
Glucose, 1	0·46	0·47	0·26
Galactose, 1	0·24	0·19	0·04
Na acetate, 0·1	0·27	0·23	.
Na glycolate, 0·1	0·00	0·02	.
Glucose (0·5 %, w/v) + substrate (%, w/v)			
Na formate, 0·1	0·36	0·00	.
Na acetate, 0·1	0·33	0·31	.
Na propionate, 0·1	0·00	0·00	.
Na butyrate, 0·1	0·00	0·00	.
Basal medium alone	0·00	0·00	0·00
At light-saturation			
Basal medium alone	0·93	0·87	0·65

Table 2. *Growth rates of* Chlorella pyrenoidosa *and* C. ellipsoidea *on sugars at different light intensities*

Growth at 25° in basal medium + 1 % (w/v) sugar. Values of specific growth rate are expressed in $\log_{10}$ unit/day

	Light intensity		
	Darkness	Weak light	Medium light
Sugar	Specific growth rate ($\log_{10}$ unit/day)		
Chlorella pyrenoidosa			
None	0·00	0·01	0·17
Glucose	0·46	0·54	0·64
Galactose	0·24	0·25	0·32
Fructose	0·00	0·05	0·17
Maltose	0·00	0·03	0·17
Sucrose	0·00	0·03	0·16
Chlorella ellipsoidea			
None	0·00	0·02	0·19
Glucose	0·47	0·54	0·64
Galactose	0·19	0·14	0·41
Fructose	0·00	0·03	0·20
Maltose	0·00	0·00	0·17
Sucrose	0·00	0·03	0·18

with the question whether the growth rate on sugar + light is more than the additive effect of sugar and light presented independently. For both strains there are possible small effects of doubtful significance. There is no evidence of any marked increase in assimilation of any of the sugars by light. Nor is there any evidence that a priming effect of light by accumulated photosynthetic products can explain the transient initial growth noted above for fructose and maltose.

Glucose and galactose presented together in darkness. Since in darkness the growth rate on glucose is about 1/2, and on galactose about 1/4, of the light-saturated growth rate, there is a possibility of additive effects of the two sugars. However, the measured growth rates on glucose and on glucose + galactose were identical. Apparently there is a common rate-limiting reaction saturated by glucose.

Sugar phosphates in darkness. Phosphoglycerate, glucose-1-phosphate, glucose-6-phosphate, fructose phosphate, and fructose-1:6-diphosphate were examined. Except for slight initial and transient effects none of these compounds supported significant growth of either of the two Chlorellas.

Organic acids in darkness. Examination of the organic acids is attended by a technical difficulty. The fatty acids are exceedingly toxic in their undissociated forms. Previous work of this laboratory (1947, unpublished) showed that for *Chlorella pyrenoidosa* photosynthesis is almost completely inhibited and glucose respiration more than 50 % inhibited by 0·004 M total acetic acid at pH 4·5. At higher pH values apparent toxicity of total acetic acid concentration decreases, but even at pH 6·7 addition of 1 % (w/v) sodium acetate inhibits growth. The substituted acids with lower p*K* values are far less toxic.

The present work was limited to examination of the following substances at 0·1 % (w/v) concentration and pH 5·6–5·7: glycine, L-aspartic acid and the sodium salts of formate, acetate, propionate, butyrate, lactate, succinate, α-ketoglutarate, pyruvate, malate, glycollate, fumarate, tartrate, malonate, and glutamate. Of these only acetate supported continued and unequivocal growth for both of the Chlorellas. Glycollate supported slight and apparently continued growth of *Chlorella ellipsoidea* but not of *C. pyrenoidosa*. The latter result is of interest in view of the observation of Tolbert & Zill (1956) that small amounts of glycollic acid, as observed with ^{14}C tracer, may be excreted and re-absorbed by *C. pyrenoidosa* during photosynthesis.

Further examination of the acids listed above at 0·1 % (w/v) concentration and with addition of 0·5 % (w/v) glucose yielded the following results as compared to glucose alone: (1) slight depression by acetate and slight or complete suppression by formate, propionate or butyrate (cf. Table 1); (2) no significant effects of other acids.

Possible growth factors. The fact that all growth rates observed in darkness were lower than the light-saturated rates leaves the possibility of a growth-factor stimulation of heterotrophic growth. Such a stimulation was sought but not found by independent additions of 0·1 % (w/v) yeast, liver, and meat-extracts and Casamino acids to 1 % glucose.

Cell-yield studies

A second criterion of growth on organic substrates can be put in terms of the maximum yield of cell material/unit quantity of substrate. It may be expressed as in studies of mould metabolism (Foster, 1949) by the *economic coefficient* (weight of organism produced × 100/weight of substrate consumed). Or, with knowledge of the composition of the cells produced, it may be expressed as % carbon assimilated (cell carbon × 100/substrate carbon consumed). It is assumed that maximum yield of cells in all cases corresponds to practically complete consumption of substrate. Further, no cognizance is taken of any extracellular organic products, although these may be expected to be small (Myers & Johnston, 1949).

As a further variable it appeared desirable to examine effects of nitrogen source on efficiency of glucose assimilation. To this end either 0·20 % (w/v) filter-sterilized urea or 0·15 % (w/v) $NH_4H_2PO_4$ + 0·125 % (w/v) tris (buffer) 2-amino-2-hydroxymethylpropane-1:3-diol were substituted for KNO_3 in the basal medium. Use of tris (buffer) as additional buffer is required to prevent decrease in pH to toxic values during assimilation of ammonia.

Table 3. *Yields of* Chlorella pyrenoidosa *and* C. ellipsoidea *grown on various carbon and nitrogen sources*

				Organisms					
Carbon Source	Nitrogen source	Volume of culture (ml.)	Amount C source (mg.)	Initial (mg.)	Max. (mg.)	Yield (mg.)	k* ($\log_{10}$/day)	Economic coefficient (%)	Carbon† assimilation (%)
Chlorella pyrenoidosa									
Glucose	Nitrate	100	500	6·5	186·6	180·1	0·44	36	43
		50	250	4·3	97·0§	92·7	0·45	37	45
		50	250	1·5	95·1	93·6	0·43	37	45
Galactose	Nitrate	50	250	4·0	81·3	77·3	0·2	31	37
Na acetate	Nitrate	50	100	3·8	13·5	9·7	0·16	10	26
Glucose	Ammonia	15	75	2·7	33·5	30·8	0·4	41	48
		50	250	2·2	108·7§	106·5	0·5	43	51
	Urea	100	500	6·6	213·9	207·3	0·43	41	50
		50	250	1·5	104·4§	102·9	0·47	41	49
C. ellipsoidea									
Glucose	Nitrate	50	250	3·6	112·8	109·2	0·5	44	53
	Urea	50	250	7·0	129·9	122·9	0·40	49	59

* Specific growth rate; varying degrees of reliability reflect differences in number of samples taken during exponential period of growth.

† Approximate: calculated for average cell product of 48 % carbon.

§ Duplicate lyophilized samples were used for the cell analyses reported in Table 4.

The pertinent data are presented in Table 3. The values of specific growth rate cited have different degrees of reliability depending upon the number of sampling points on which they are based. Internal comparisons can be made in terms of the economic coefficient, or in equivalent terms of approximate % carbon assimilated.

Glucose versus galactose. It is difficult to visualize a model which will explain why galactose should be assimilated less completely than glucose. However, it will be noted that for galactose the specific growth rate is much lower. Consumption of a given quantity of substrate requires a longer time period for galactose, attended by a greater fractional contribution to basal or maintenance metabolism. Simplification of the growth curve such as Fig. 2A to an exponential curve breaking sharply at the maximum, the integrated area under the curve $(e^{k't}-1)\,N_0/k'$ gives the mg.-hr. of cells required to consume the substrate. For consumption of 100 mg. substrate carbon the above calculation yields 2230 mg.-hr. for glucose and 4700 mg.-hr. for galactose. (Actually the figure of mg-hr. for galactose is higher than calculated since the terminal growth rate did not break sharply but fell slowly in approach to zero.) It appears that a greater contribution to maintenance metabolism can explain the less complete assimilation of galactose. It follows as a general principle that substrates which support lower growth rates may show lower efficiency of assimilation because of necessarily greater contribution to maintenance metabolism.

Glucose versus acetate. Experiments were limited to low initial quantities of acetate because of toxic effects at higher concentrations. The markedly less complete assimilation of acetate is to be expected for reasons besides that presented above for galactose; a less complete assimilation of acetate to starch has been found in manometric studies of oxidative assimilation in starved cells of *Chlorella pyrenoidosa* (Myers, 1947).

Chlorella pyrenoidosa *versus* C. ellipsoidea. Comparison of the two species shows a clearly more complete assimilation of glucose by *C. ellipsoidea* than by *C. pyrenoidosa.*

Effect of nitrogen source. As compared with nitrate, both ammonia and urea allowed more complete assimilation of carbon from glucose. This was to be expected as a consequence of the energy demand for nitrate reduction; it is explored further in terms of the more complete data on cell analysis presented below.

Effect of added substrates. The possible utilization of other substrates during glucose assimilation may be tested by adding the substrate and examining whether there is any increase in carbon assimilation as calculated for the glucose carbon. This was done with *Chlorella pyrenoidosa* for fructose, maltose, sucrose and glycollic acids by additions of 0·5 % (w/v) of each substrate to 0·5 % (w/v) glucose. No increase in the economic coefficient for glucose utilization was observed in any case. The result provides convincing evidence that these substances are not utilized even during glucose assimilation.

Efficiency of cell synthesis

Efficiencies of conversion and comparison of effects of nitrogen source can be obtained from elementary analyses on three of the samples noted in Table 3; the salient results are presented in Table 4. From the elementary analyses, taking oxygen by difference and calculating to an ash-free organic substance basis, the cell compositions may be expressed as molecular formulae which

Table 4. *Cellular composition and derived values for* Chlorella pyrenoidosa *grown in darkness on glucose*

Nitrogen source	Elementary analysis (%)				Ash-free unit formula	Formula weight	Carbon recovery (%)	$\Delta H_{comb.}$ (kcal./mole)	Efficiencies (%)	
	C	*H*	*N*	Ash					Rubner coefficient	correction for N source
Nitrate	48·04	7·06	8·39	4·56	$C_{6\cdot68}H_{11\cdot69}O_{3\cdot34}N$	159·3	44·7	848	50·6	56·4
Ammonia	49·13	7·12	6·41	5·05	$C_{8\cdot94}H_{15\cdot56}O_{4\cdot41}N$	207·4	51·4	1136	58·2	58·2
Urea	47·93	7·24	7·46	6·43	$C_{7\cdot51}H_{13\cdot60}O_{3\cdot63}N$	175·8	49·5	971	57·0	57·2

here are reduced arbitrarily to unit nitrogen. If it be assumed that the metabolic carbon products are only cells and carbon dioxide, an assumption correct to better than 90 % (Myers & Johnston, 1949), the following balanced equations may be written to express over-all metabolism.

On nitrate:

$$HNO_3 + 2\cdot49C_6H_{12}O_6 + 5\cdot78O_2 \rightarrow C_{6\cdot68}H_{11\cdot69}O_{3\cdot34}N + 8\cdot28CO_2 + 9\cdot60H_2O. \quad (1)$$

On ammonia:

$$NH_4OH + 2\cdot90C_6H_{12}O_6 + 7\cdot28O_2 \rightarrow C_{8\cdot94}H_{15\cdot56}O_{4\cdot41}N + 8\cdot46CO_2 + 11\cdot62H_2O. \quad (2)$$

On urea:

$$0\cdot5N_2H_4CO + 2\cdot53C_6H_{12}O_6 + 6\cdot83O_2 \rightarrow C_{7\cdot51}H_{13\cdot60}O_{3\cdot63}N + 8\cdot16CO_2 + 9\cdot38H_2O. \quad (3)$$

The R.Q. values predicted from equations (1) and (2) check reasonably the values obtained manometrically (Cramer & Myers, 1948).

The heats of combustion of the cells produced may be obtained by determining the number of moles of oxygen required for combustion to CO_2, H_2O and N_2 and taking some figure as the calorie equivalent for one mole of oxygen. The latter figure carries uncertainty within the range 103–113 kcal./mole. In calculations of photosynthetic efficiency a value of 112 kcal./mole is properly chosen since the product is assumed to be carbohydrate. However, for production of total cell material the product is only partly carbohydrate. The problem was examined by Kok (1952) who obtained both calorimetric measurements and cell analyses on cells of a Chlorella grown photosynthetically. Unfortunately, the results were obtained upon parallel samples which differed in ash content. Kok chose to ignore the differences in ash content and estimated an equivalent of 112 kcal./mole O_2. However, when Kok's data are calculated to an ash-free basis and the calorimetric contribution of the ash fraction is considered negligible, then the equivalent becomes 107 kcal./mole O_2.

An independent means of calculating a calorie equivalent for oxygen is provided by the data of Kharasch (1929). Cited values of heat of combustion/mole for various organic compounds may be divided by the computed mole O_2/mole, required for combustion. For example, the 673 kcal./mole heat of combustion of glucose divided by 6 mole O_2/mole gives a calorie equivalent of 112·2 kcal./mole O_2. For the carbohydrates glucose, sucrose, starch and glycogen the equivalents are in the range 112–113 kcal./mole O_2. For the lipids glyceryl-trilaurate, palmitic acid, and stearic acid, the equivalents range from 104 to 105 kcal./mole O_2. For the amino acids and peptides aspartic, glutamic, glycine, leucine, phenylalanine, tyrosine, glycylglycine and triglycylglycine, the equivalents range from 103 to 105 kcal./mole O_2. Using the procedure of Spoehr & Milner (1949), the average composition of our cells is calculated as 49 % (w/w) protein, 36 % (w/w) carbohydrate, and 15 % (w/w) lipid. From the corresponding oxygen equivalents of protein 104, carbohydrate 112·5, and lipid 104·5 kcal./mole O_2, respectively, the weighted average for our cells becomes 107 kcal./mole O_2 which is taken for calculation of the heats of combustion shown in Table 4.

Efficiencies of metabolic conversions have been variously calculated depending upon the premises and intent of the calculation (cf. Burk, 1931; Winzler & Baumberger, 1938). As a starting-point we adopt the classical Rubner coefficient (cf. Foster, 1949). Output work performed is taken as the energy stored in the cells produced, in terms of their heat of combustion. Input energy expended is taken as the heat of combustion of the substrate consumed. For example, the efficiency for equation (1) is $848/(2{\cdot}49 \times 673) = 0{\cdot}506$. Calculated values are listed in Table 4. It will be noted that the efficiency so obtained for cell synthesis from nitrate is significantly lower than that obtained for similar synthesis from ammonia. However, it is clear that additional work is required for reduction of nitrate and that this should be reckoned as part of the output work performed. If it be assumed that cell synthesis starts from nitrogen in a form energetically equivalent to NH_3, then equation (1) includes work done in the reaction

$$HNO_3(aq.) + 2H_2O(l) \rightarrow NH_4OH(aq.) + 2O_2, \quad \Delta H = +98{\cdot}4, \qquad (4)$$

and equation (3) includes the work done in the reaction

$$0{\cdot}5N_2H_4CO(aq.) + 1{\cdot}5H_2O(l) \rightarrow NH_4OH(aq.) + 0{\cdot}5CO_2(aq.), \quad \Delta H = +3{\cdot}6. \qquad (5)$$

For example, the corrected efficiency for equation (1) becomes $(848+98)/(2{\cdot}49 \times 673) = 0{\cdot}564$. Efficiency values of Table 4 thus corrected are not significantly different for the different nitrogen sources, although exact identity would imply 100 % efficiency for nitrate reduction.

DISCUSSION

From the accumulated data on heterotrophic growth of *Chlorella pyrenoidosa* the nutritional pattern becomes evident. Its cellular synthesis proceeds with high efficiency but only from a very limited number of substrates. Lack of versatility in the use of organic substrates appears to be a result of permeability restrictions. Sucrose, which will not serve as a carbon source for growth, has been identified as an intracellular component (Milner, 1948). Likewise many of the other organic compounds which fail to support growth have been identified as intermediary metabolites by tracer studies (Calvin *et al.* 1951).

The maximum rate of cell synthesis provided by light saturated photosynthesis is not increased by addition of glucose (Killam & Myers, 1956). At low light intensities the rates supported by photosynthesis and glucose assimilation become approximately additive. However, the rate supported by glucose in darkness is not augmented by addition of the other utilizable substrates galactose and acetate. A simple hypothesis will encompass these observations: there is a common reaction in cell synthesis from all three substrates which is rate-saturated during glucose assimilation; in photosynthesis this reaction is by-passed by an alternative pathway or is not required.

It is instructive to compare the 58 % efficiency of total cell synthesis from glucose with the efficiency observed for the simpler synthesis of storage carbohydrate alone. The latter has been measured manometrically in starved cells at energy efficiencies which calculate to 83 and 88 % as reported for *Chlorella pryenoidosa* (Myers, 1947) and *C. vulgaris* (Syrett, 1951). Further extension may be made to a comparison of the efficiencies of heterotrophic and autotrophic metabolism. For total cell synthesis from carbon dioxide in light an approximate efficiency of 20 ± 2 % may be taken from the data of Kok (1952) and Oorschot (1955); slightly lower values are being obtained currently in our laboratory. For carbohydrate synthesis from carbon dioxide (photosynthesis) there is a wide scatter of efficiency values corresponding to quantum yields ranging from about 1/4 to 1/10 (Rabinowitch, 1951). A quantum yield of 1/8, observed at 6800Å. where the quantum energy is 42 kcal/Einstein, would correspond to a thermodynamic efficiency of 33 %. The last figure cannot be compared rigidly with the other three, not only because of uncertainty as to its exact value, but also because its determination includes correction for respiration or maintenance metabolism not applied in the other cases. However uncertain the comparison may be as applied to photosynthetic efficiency, an important requirement is clearly demonstrated: if photosynthesis is taken to represent synthesis of carbohydrate alone, then its efficiency must be determined under conditions which rigidly preclude other cellular syntheses.

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The Smooth and Rough Somatic Antigens of *Pasteurella pseudotuberculosis*

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SUMMARY: From strains of *Pasteurella pseudotuberculosis* representing each of the five serological groups and some 'Rough' forms, lipopolysaccharides were isolated after disintegration of acetone-dried saline extracted organisms with phenol. The materials appear to carry the specificities on which the serological classification of the species is based; they are relatively poor antigens when isolated but antigenicity can be enhanced by combining with lipoprotein. Analyses show some unusual features, an aldoheptose sugar being present in all of the materials and some of the 'Smooth' cell products contain dideoxy sugars; rhamnose is absent. The 'Rough' cell products are somewhat similar in composition to the 'Rough' somatic polysaccharide of *Pasteurella pestis*, to which they are also related serologically.

The principal antigenic features and the basis of the present serological classification of *Pasteurella pseudotuberculosis* were established by Schütze (1928, 1932). Strains were classified into three serological groups by bacterial agglutination, heat-stable Smooth 'O' somatic antigens analogous to those of the Salmonellas being postulated to account for this. Another heat-stable component was common to all 'Smooth' and 'Rough' strains of *P. pseudotuberculosis* and extended also to *P. pestis*; this was attributed to a Rough 'R' somatic antigen. Flagellar 'H' antigen was common to all *P. pseudotuberculosis* strains but absent from *P. pestis*.

This serological work was amplified by Thal (1954); a large number of strains (186) were allotted to their serological groups and only two could not be accommodated in groups I–III of Schütze (1928). These also differed from one another and were classified in two new groups IV and V. It is well known that certain strains of *Pasteurella pseudotuberculosis* produce a powerful heat-labile toxin and such strains were shown to be confined to group III. This exotoxin differs from that of *P. pestis* (Schar & Thal, 1955). The group IV strain differed from all other 'Smooth' strains in being avirulent, but it is of interest to note that when injected alive into guinea pigs this group IV strain was able to protect against infection by members of other serological groups and also against plague infection (Thal, 1956). Rough avirulent strains obtained from different groups could not be distinguished serologically from each other since they possessed the 'Rough' somatic specificity present also in the Smooth forms and in *P. pestis*.

This general picture appears to be very similar to the situation found among the Enterobacteriaceae but no antigens have been isolated having the properties described. Extraction of *Pasteurella pseudotuberculosis* cells with trichloroacetic acid (Boivin & Mesrobeanu, 1935; Girard, 1941), or with diethyleneglycol (Schar & Thal, 1955) failed to yield an endotoxin ('O' somatic antigen). Some work in this laboratory is being directed towards a clarification of such serological findings by chemical characterization of the substances responsible. In the Pasteurella group the 'R' somatic antigen of *P. pestis* has already been described (Davies, 1956); this substance can be extracted from any strain whether rough or smooth, virulent or avirulent, toxic or non-toxic, etc., which confirms Schütze's postulate that this species is basically 'Rough' in the Salmonella sense. Evidence for the individuality of a number of other antigens has been given by Crumpton & Davies (1956*a*) who showed that six antigens were common to *P. pseudotuberculosis* and *P. pestis*; one of these has now been described in more detail (Crumpton & Davies, 1957) and others are being examined.

In spite of the failures to extract typical 'endotoxins' the heat stability of the *Pasteurella pseudotuberculosis* group specific components does suggest the presence of polysaccharides and the inability to detect group specific proteins (Crumpton & Davies, to be published) supports this view. Lazarus & Nozawa (1948) obtained toxic phage lysates but there was no indication as to the nature of the toxic substance. Thal (1954) was able to obtain group specific precipitin reactions using warm saline extracts of agar grown cells.

In the present paper the isolation and some properties of the 'Smooth' group specific and 'Rough' somatic antigens are described; their serological properties are dealt with more fully in the following paper (Crumpton, Davies & Hutchison, 1958).

METHODS

Organisms. Strains belonging to each of the five serological groups of *Pasteurella pseudotuberculosis* were very kindly supplied by Dr Thal (Veterinärmedizinischen Staatsinstitut, Stockholm). These were maintained on agar slopes (tryptic meat digest agar with peptic sheep's blood). Rough strains of certain groups were isolated from plates by using the same medium. The serological groups numbered I, II, III, ..., by previous workers have, in accordance with the recommendations of the International Bacteriological Code of Nomenclature, been referred to herein by letters A, B, C, ... Thus, for example, the Smooth state of strain number 25 in group V and the Rough state of strain number 74 from group I (Thal, 1954) are designated 25/E/S and 74/A/R, respectively.

Growth and recovery of organisms. Bacteria were grown in tryptic meat broth containing 1% (w/v) galactose (Ross, Hakes & Herbert, private communication); 250 ml. amounts of medium were dispensed in 'Thompson' bottles (Gallenkamp and Co., London, E.C. 4). These were inoculated with 1 ml. (1 mg. dry wt. organisms) of a saline suspension of the given organism which had been grown for 18 hr. on agar slopes. Incubation for 40 hr. on a reciprocating shaker at 37° gave yields varying from 3 to 8 g. dry wt./l. in different cases. Organisms were recovered by centrifuging at 20,000 g, resuspended in a small volume of cold water and poured into 10 vol. of acetone at $-20°$. After several changes of cold acetone the organisms were dried *in vacuo* over H_2SO_4.

Materials for analysis. Samples of extraction products for analysis were thoroughly dialysed against distilled water at 0–2° and dried from the frozen state. They were further dried to constant weight *in vacuo* at 78° before making into aqueous solutions of known concentration.

Nitrogen and phosphorus estimations. Nitrogen was determined by the micro-Kjeldahl method, using the distillation apparatus of Markham (1942); phosphorus was estimated on samples containing 5–30 μg. P by a modification of the method of Fiske & Subbarow (1925).

Sugars. Hexosamine was measured by the Elson & Morgan (1933) method and aldoheptose according to Dische (1953).

Chromatography. Polysaccharide samples were hydrolysed in sealed ampoules at 100° with $\text{N-}H_2SO_4$ for 15 hr. and neutralized with $Ba(OH)_2$. The neutral salt-free solutions were evaporated to dryness *in vacuo* and redissolved at suitable concentrations for application to paper chromatograms. Whatman No. 1 paper was used with a variety of solvent systems (Davies, 1957*a*); papers were sprayed with Ehrlich's reagent (Partridge, 1948) to reveal 2-amino sugars, or with anisidine hydrochloride (Hough, Jones & Wadman, 1950) which distinguishes by colour the different classes of reducing sugars.

Nucleic acid. The nucleic acid content of extraction products was estimated by measuring in a Unicam ultraviolet spectrophotometer the absorption at 260 mμ. of material dissolved in 0·05 N-NaOH. A purified preparation of ribonucleic acid (RNA) was used as a standard for reference.

Ultracentrifugation. High-speed centrifuge runs were carried out in a Spinco Model 'L' preparative ultracentrifuge and analytical runs in a Spinco Model 'E' machine.

Antisera. Antisera were prepared in rabbits by the intravenous injection of extraction products dissolved in sterile saline. Injections were given weekly until examination of serum samples indicated that the required antibodies had been formed. Sera against whole living organisms were prepared by similar methods in white rats, which are not susceptible to *Pasteurella pseudotuberculosis* infection.

Precipitation reactions. Antigens and antisera were examined by the agar diffusion precipitin technique of Ouchterlony (1953) as used by Crumpton & Davies (1956*a*). Precipitation in fluid medium was carried out as described by Davies (1956).

Toxicity and pyrogenicity. Tests were performed according to Davies, Morgan & Mosimann (1954) and Davies (1956), respectively.

RESULTS

Saline extraction

Extraction of acetone-dried organisms with water yielded material very largely protein in nature. More complete extraction of the surface proteins could be made with saline (2·5 %, w/v) as used by Baker *et al.* (1952). Batches of organism (*c.* 50 g.) of each of five 'Smooth' strains and four 'Rough' strains were suspended in saline (1 l.) and stirred for 24 hr. at 20°, toluene being added to prevent mould and bacterial growth. Suspensions were centrifuged at

12,000 *g* for 2 hr. and the sediment resuspended in water, poured into 10 vol. of acetone at −10° and subsequently dried. The saline supernatant solutions were dialysed until free from salt and freeze dried; the products were preserved for study of the protein antigens they contained, which were mainly antigens 2, 4, 5, 6 and 7 (Crumpton & Davies, 1956*a*); these will be described in subsequent communications. These extracts contained little carbohydrate and nucleic acid amounted to not more than 5%.

Phenol extraction

Samples of organism recovered after saline extraction were treated with diethyleneglycol (Morgan, 1937; Morgan & Partridge, 1940; Davies *et al.* 1954), with aqueous pyridine (Goebel, Binkley & Perlman, 1945), with aqueous glycerol (Binkley, Goebel & Perlman, 1945) or phenol (Westphal, Lüderitz & Bister, 1952). Only in the phenol extracts were significant yields of carbohydrate obtained.

The bulk of the organisms recovered from each saline extract were therefore extracted by the phenol method, being suspended at 10% (w/v) concentration in water and heated to 65°. An equal vol. of phenol (90%, w/v) at the same temperature was added and the single phase system stirred at this temperature for 20 min. Suspensions were then cooled to 2° and centrifuged at this temperature to hasten the separation of water and phenol phases. The small amount of sediment obtained was composed of protein and DNA. The phenol phase contained denatured protein and the aqueous phase contained soluble carbohydrate and degraded RNA. The aqueous phase was poured into 10 vol. of acetone at −10° and the precipitated material redissolved in water and precipitated by adding 2 vol. ethanol. This precipitate was redissolved at *c.* 1% (w/v) concentration and centrifuged at 100,000 *g* for 4 hr. Degraded RNA was thus left in the supernatant fluid and the sedimented material was redissolved and centrifuged in the same way until the ultraviolet adsorption of a sample showed the nucleic acid content to be no more than 1–2%. The final sedimented fractions proved to be lipopolysaccharides which differed considerably in solubility from different strains, so that some of the relatively insoluble ones (e.g. 32/D/R) were obtained in better yield and quite free from nucleic acid. The more soluble preparations (e.g. 27/C/S) were obtained in smaller yield, a considerable amount of polysaccharide remaining in the nucleic acid fraction.

Nucleic acid was recovered from the centrifuged supernatant fluids by dialysis and freeze-drying. Further amounts of polysaccharide could be recovered from these fractions by other methods (ethanol fractionation, acid precipitation, ribonuclease), but the original centrifuge sediments recovered by dialysis and freeze-drying were used in the present work. Some data for these materials are shown in Table 1. The weights recorded are not related to true yields with respect to cell content, since in all cases some polysaccharide remained in the nucleic acid fractions. The actual yields of lipopolysaccharide in the aqueous phase of the phenol extracts was estimated to be in the range of 1–3% of the dry weight of organism.

Table 1. *Lipopolysaccharides obtained by phenol extraction of* Pasteurella pseudotuberculosis *strains*

Organism (strain no./group/ colony form)	Wt. (mg.)	N (%)	P (%)
14/A/S	365	2·2	1·6
16/B/S	334	2·0	1·3
27/C/S	76	1·8	1·6
32/D/S	343	2·3	1·5
25/E/S	986	1·5	1·4
16/B/R	187	3·1	1·5
32/D/R	745	2·2	1·7

Carbohydrate fractions

Composition. By analogy with materials, of similar N and P content and other properties, previously obtained from various Gram-negative organisms by the phenol extraction method (e.g. Westphal *et al.* 1952), the products were expected to be lipopolysaccharides. This was checked with two samples (25/E/S and 32/D/R) which were degraded by heating with dilute acetic acid as already described (Davies, 1955). The acetic acid-insoluble material which separated was also insoluble in ether but dissolved in chloroform and appeared to be entirely analogous to the chloroform-soluble phospholipids which have been fully described (Westphal & Lüderitz, 1954) from similar extracts of various species of Enterobacteriaceae. From the acetic acid supernatant solution a degraded polysaccharide hapten could be recovered by precipitation with ethanol.

Homogeneity. The presence of less than 2% nucleic acid could be demonstrated in the lipopolysaccharides by examination of their ultraviolet absorption spectra; ribose was not detected on paper chromatograms when hydrolysates were examined for their sugar constituents as described below. The absence of free protein follows from the method of extraction wherein such material is denatured by warm phenol. This was confirmed by failure of the products to give any protein reactions, their low N content similar to that of better characterized lipopolysaccharides (Davies, 1956), the presence of only traces of ninhydrin reactive material other than amino sugar in hydrolysates examined on paper chromatograms and which can be accounted for as products of the degradation of the lipid moiety, and lack of ultraviolet absorption in the region of 280–290 mμ.

Owing to the poor solubility of lipopolysaccharides extracted by the phenol method, demonstration of physical homogeneity is extremely difficult. Solubility in salt solution is poorer than in water, but in cases where electrophoretic examination has been possible (Davies, Morgan & Record, 1955) there has clearly been a very small net charge. It therefore seemed permissible to carry out ultracentrifuge runs in water, this was done in the present work with the more soluble preparations which sedimented as single components.

Degradation with acetic acid has been referred to above, the extent depending upon the types of glycosidic linkages present and varying with different materials (Davies *et al.* 1955; Davies, 1956). It might therefore be expected that a mixture of lipopolysaccharides which failed to resolve on ultracentrifugal analysis might be separable after such degradation. A degraded polysaccharide hapten preparation obtained from 25/E/S lipopolysaccharide by treatment with 1 % (w/v) acetic acid at 100° for 3 hr. in a nitrogen atmosphere, and subsequent separation from the phospholipid component, was examined in the analytical ultracentrifuge, and a single sedimentation boundary was revealed. These results were quite insufficient evidence of homogeneity but were possible indications in that direction.

Important for the purposes of the present work was an indication of whether the materials were immunologically homogeneous. A suggestion that this might be the case was deduced from gel diffusion experiments. Each lipopolysaccharide reacted with a serum prepared by immunization with organisms of the homologous strain, and in each case a single line of precipitation was formed. These sera contained antibodies which reacted with all the major antigenic components of the original organisms. Sera prepared by injecting lipopolysaccharide which had been combined with lipoprotein (Partridge & Morgan, 1940; Davies, 1956) reacted with the homologous lipopolysaccharide and also with whole organisms of the homologous serological group, to give only one line of precipitation. With the five 'Smooth' group products the five lines indicated different specificities since they were in no instance contiguous when reacting from adjacent reservoirs. No cross-reaction was detected on diffusion plates between 'Smooth' and 'Rough' products nor between 'Smooth' *Pasteurella pseudotuberculosis* polysaccharides and *P. pestis* 'Rough' somatic polysaccharide.

Chromatographic analysis. The sugar constituents of lipolysaccharides extracted from the various groups are shown in Table 2. Estimates of the amount of aldoheptose present are in terms of D-glycero-L-mannoheptose (Davies, 1957*a*); in the method of estimation used different heptose sugars give different amounts of absorption and a true estimate can be made only when the identity of a sugar is known. The chromatographic behaviour of the heptose component was the same for each of the polysaccharides shown in Table 2, but although this resembled D-glycero-L-mannoheptose or its optical enantiomorph in some respects, its true identity is not yet established. Estimates of total amino sugar are shown in Table 2, it should be noted that glucosamine is not necessarily a component of the polysaccharide moiety since it is known to be a constituent of the phospholipid.

Biological properties

Antigenicity. Although antibodies to the polysaccharides can readily be obtained by immunizing rabbits with killed whole organisms, or by injecting rats with living organisms, the isolated lipopolysaccharides were poorly antigenic. Their antisera failed to precipitate the homologous materials in fluid media and gave no line of precipitation when tested by the agar diffusion precipitin method. A definite antigenic response was, nevertheless, detected by the more sensitive haemagglutination technique with red cells sensitized with the corresponding polysaccharide as described by Crumpton *et al.* (1958). Precipitating antisera were obtained by injecting lipopolysaccharide combined with the lipoprotein component of the *Shigella dysenteriae* somatic antigen as used by Davies (1956). This method was used for each of the materials extracted and gave monospecific antisera, whereas in the sera prepared in rats by immunization with living organisms the main response had clearly been directed against protein antigens (Crumpton & Davies, to be published).

Table 2. *Sugar constituents of* Pasteurella pseudotuberculosis *polysaccharides*

Organism (strain no./group/ colony form)	Galactose	Glucose	Mannose	Fucose	Tyvelose	Abequose	Glucosamine	Galactosamine	Heptose	Heptose* content (%)	Hexosamine content (%)	Unidentified† components
14/A/S	+	+	.	.	.	.	+	.	+	5	4·3	1
16/B/S	+	+	+	.	.	+	+	.	+	17	9·5	.
27/C/S	.	+	+	+	.	.	+	+	+	13	5·0	1
32/D/S	.	.	+	.	+	.	+	+	+	19	6·3	.
25/E/S	.	+	+	+	+	.	+	+	+	18	7·5	.
74/A/R	.	+	.	.	.	.	+	.	+			.
16/B/R	.	+	.	.	.	.	+	.	+	5	5·0	.
43/C/R	.	+	.	.	.	.	+	.	+	17		.
32/D/R	.	.	.	.	.	.	+	.	+	26	9·5	.

* Estimated as D-glycero-L-mannoheptose.
† Possibly dideoxy sugars.

With lipopolysaccharide + lipoprotein induced antisera against the products of each of the 5 groups and also 2 Rough strains, each material was tested by tube precipitation against each serum. These results are shown in Table 3. Each serum reacted most strongly with its homologous lipopolysaccharide, and the group specificities appear to be carried by these materials. Considerable cross-reactions occurred, however, especially with the Rough strain products. In some instances stronger precipitation was found in heterologous reactions

Table 3. *Precipitin reactions of* Pasteurella pseudotuberculosis *lipopolysaccharides*

Sera were prepared by immunization of rabbits with isolated lipopolysaccharides combined with 'Shiga' lipoprotein. Results are expressed as the sum of up to 3 points for degree of precipitation in each tube showing a reaction. Serum undiluted, lipopolysaccharide in serial 1/2 dilutions beginning at 1/7260.

Antigen group	Serum group						
	A/S	B/S	C/S	D/S	E/S	B/R	D/R
14/A/S	5	2	0	5	3	2	2
16/B/S	0	13	0	8	4	3	5
27/C/S	0	3	6	6	3	2	3
32/D/S	1	3	0	14	6	3	3
25/E/S	0	1	0	4	11	1	3
16/B/R	1	5	0	6	4	4	4
32/D/R	2	6	0	10	5	4	8

where the homologous serum was relatively weak. Some Rough strain products also precipitated with *Pasteurella pestis* antisera.

Because of difficulty in interpreting these results and also on account of the poor solubility of some of the preparations, a more extensive serological examination was carried out by the more sensitive and versatile haemagglutination method. These results are recorded in the following paper (Crumpton *et al.* 1958).

Toxicity and pyrogenicity. Like the somatic antigens prepared from members of the Enterobacteriaceae, the *Pasteurella pseudotuberculosis* lipopolysaccharides are toxic for mice, but whereas the former generally have a LD 50 dose in the range of 20–100 μg. the present materials had a LD 50 dose of about 500 μg. There was a suggestion, however, that effective toxicity was dependent to some extent on state of dispersion since the LD 50 dose was decreased to 200–300 μg. after heating solutions at 80° for 5 min. This heat treatment gave a stable opalescent solution of those materials which were otherwise unstable cloudy suspensions. A similar effect was noted with respect to pyrogenic potency. Lipopolysaccharide 32/D/R was particularly insoluble and was pyrogenic in rabbits at 1 μg./kg. rabbit; after heat treatment a significant pyrogenic response was obtained at 0·005 μg./kg. rabbit, a figure more in keeping with those of other pyrogens.

DISCUSSION

In the course of examining the antigens of *Pasteurella pestis* it was observed that the organism shared a number of antigens with *P. pseudotuberculosis*, and most of these were already known to be proteins (Crumpton & Davies, 1956*a*). When *P. pseudotuberculosis* strains of different serological groups were compared by the agar diffusion precipitin method no differences were found between the protein antigen line patterns which would account for the serological grouping. This agreed with the observations of Schütze (1928) that heat-stable antigens were responsible for the serological group specificities. It was noted, however, that all avirulent strains, whether rough or smooth, lacked one protein antigen (Crumpton & Davies, 1957).

The presence of surface protein antigens, as, for example, in *Pasteurella pestis*, makes the extraction of polysaccharides more difficult (Davies, 1956); the same situation exists in *P. pseudotuberculosis* where the somewhat drastic phenol method was finally chosen. The products of this extraction method are poor antigens but their serological specificity seems unimpaired; they are variable in their state of aggregation and it should be noted that this affects the measurement of some biological activities. A disadvantage of using poorly soluble materials of this type is that a satisfactory demonstration of physical homogeneity is precluded, and with the amounts of fractions available only circumstantial evidence of homogeneity has been obtained. However, there are indications that 'Rough' lipopolysaccharide may not be present in smooth organisms. The sugars present in the 'Rough' products are also present in the 'Smooth' products, but this is clearly not contamination since some of the latter contain, for example, more aldoheptose sugar and glucosamine than some of the former. The 'Rough' polysaccharides are themselves only qualitatively similar, and their close constitutional relationship to *P. pestis* polysaccharide is of interest. It seems likely, therefore, that the 'S' and 'R' antigens of Schütze (1932) and Thal (1954) are not discrete chemical entities, but that the 'R' somatic specificity is due to a part of the structure of the 'S' antigen. The view of Schütze (1932) that *P. pestis* shares a common 'R' somatic specificity with *P. pseudotuberculosis* could also be reconciled with this interpretation.

A situation found in *Shigella dysenteriae* may not be dissimilar from that in *Pasteurella pseudotuberculosis*. The 'O' somatic antigen contains galactose, glucosamine and rhamnose (Morgan, 1938); no cross-reaction occurs between rough and smooth cell products but a 'Rough' polysaccharide has now been described (Davies, 1957*b*) which contains galactose, glucosamine and an aldoheptose. No 'Rough' component could be detected in smooth organisms which again suggests that the S→R change reflects a change in polysaccharide constitution and not the loss of a 'Smooth' polysaccharide to reveal beneath a substance similar to that exposed on the surface of rough organisms. It is noteworthy that in the Enterobacteriaceae virulent organisms usually possess polysaccharides which contain deoxy sugars (rhamnose, fucose, tyvelose or abequose), these are not present in avirulent forms. This is also true of the Pasteurellas studied except that *P. pestis* does not produce such a sugar, and *P. pseudotuberculosis* 32/D/S does so. In the latter it is known, however, that an important protein antigen is missing (Crumpton & Davies, 1957), while in the former protein antigens known to be important in pathogenicity are produced (Crumpton & Davies, 1956*a*, Burrows & Bacon, 1956).

It might be noted that at the time of writing all polysaccharides extracted from rough forms of Gram-negative bacteria and which have been adequately analysed have proved to contain aldoheptose sugars. The identity of sugars recorded in Table 2 should be regarded as provisional since the information is based, apart from amino sugar and heptose, on chromatography alone. This is insufficient for final identification, more particularly in view of the number of new sugars which have recently been identified (e.g. Westphal & Lüderitz, 1953; Strange, 1956; Crumpton & Davies, 1956*b*; van Tamelen *et al.* 1956; MacLennan & Davies, 1956; Muir, 1957; Fouquey, Polonsky & Lederer, 1957). The dideoxy sugars in two instances could not be identified with any confidence. The absence of rhamnose is noteworthy, this sugar being widely distributed in bacterial polysaccharides.

Since this work was completed, the sugar constituents of a polysaccharide obtained from an unspecified strain of *Pasteurella pseudotuberculosis* have been reported by Sasaki (1957). This material differed in all its main features from the lipopolysaccharides described herein: xylose, arabinose and rhamnose were recorded, sugars notably absent from the preparations described above, whereas tyvelose, abequose and aldoheptose were not detected.

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The Serological Specificities of *Pasteurella pseudotuberculosis* Somatic Antigens

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SUMMARY: The serological specificities of lipopolysaccharides obtained from representatives of each of the five serological groups of *Pasteurella pseudotuberculosis* have been examined by haemagglutination methods. At concentrations of 500 µg./ml. solutions of the lipopolysaccharides failed to give full sensitization of erythrocytes, but after treatment with alkali full sensitization was obtained at concentrations in the range of 50 to 3 µg./ml. By using antisera prepared in rats by immunization with living *P. pseudotuberculosis*, the capacity of each 'Smooth' lipopolysaccharide to inhibit the agglutination of sensitized erythrocytes by each group antiserum was determined. Absolute group specificity was found by this method. It is considered that the lipopolysaccharides bear the specificity of the somatic antigens on which the serological grouping of the species is based. 'Smooth' *P. pseudotuberculosis* lipopolysaccharides failed to inhibit the agglutination by *P. pestis* antisera of erythrocytes sensitized with *P. pestis* lipopolysaccharide, but inhibition of this system was obtained with lipopolysaccharides of 'Rough' *P. pseudotuberculosis* strains derived from various serological groups. These 'Rough' lipopolysaccharides were closely related serologically to one another and to the *P. pestis* product but were not identical in specificity. It is considered that in Smooth *P. pseudotuberculosis* strains the 'Smooth' somatic antigen does not overlie a 'Rough' somatic antigen.

In the Smooth state of *Pasteurella pseudotuberculosis* heat-stable somatic antigens have been detected, characteristic of one of the five serological groups into which this species has been subdivided (Arkwright, 1927; Schütze, 1928; Kauffmann, 1932; Thal, 1954). It has been considered that in 'Smooth' cultures a 'Smooth' somatic antigen overlies a 'Rough' somatic antigen which is exposed on the surface of 'Rough' organisms and has a specificity common to all 'Rough' cultures derived from any of the serological groups. The specificity of the 'Rough' component is also common to all strains of *P. pestis* (Schütze, 1932; Bhatnagar, 1940; Thal, 1954, 1956). These conclusions were based on precipitation, agglutination, serum absorption and immunological studies with whole organisms or heated autolysed culture filtrates.

It has been suggested that the 'Rough' somatic antigen common to *Pasteurella pseudotuberculosis* and *P. pestis* may be composed of two serologically distinct components (Chen & Meyer, 1955; Bhagavan, Chen & Meyer, 1956); this idea was based on the results of diffusion-precipitin analysis. By this method Crumpton & Davies (1956) showed that the two species possessed at least 7 antigens in common, but heat stability with respect to antigenicity, as opposed to haptenic reactivity, could be clearly demonstrated for only one of these components when antisera against cultures of Rough *P. pseudotuberculosis* and *P. pestis* were compared (Crumpton & Davies, to be published). This heat-stable component was isolated from *P. pestis* and purified by Davies

(1956); it is considered to be the 'Rough' somatic antigen postulated by Schütze (1932).

Somatic antigens derived from strains representing each of the serological groups of *Pasteurella pseudotuberculosis* have been extracted and purified (as lipopolysaccharides), and described by Davies (1958). These materials did appear to carry group specificity, but in precipitin tests they cross-reacted in some instances with one another and also with analogous products of 'Rough' strains. Nevertheless, the indications from chemical and physical data were that the materials were single chemical entities.

Of the methods available for studying the serological properties of polysaccharides the 'indirect bacterial hemagglutination' method (Neter, 1956) has certain distinct advantages. In this test material is adsorbed on to erythrocytes which thus acquire the specificity of the adsorbed substance. This overcomes difficulties associated with the rather poor solubility of lipopolysaccharides extracted by the phenol method (Westphal, Lüderitz & Bister, 1952); alternative methods of extraction are not very satisfactory where surface protein antigens are present. The haemagglutination method has also proved in many instances to be the most sensitive one for the detection and titration of antibodies (Neter *et al.* 1955) and in modified form can be used for the detection of very small amounts of material (Neter & Gorzynski, 1954). In the present work the haemagglutination and haemagglutination inhibition methods have been used to examine the serological specificities of the *Pasteurella pseudotuberculosis* 'Smooth' and 'Rough' lipopolysaccharides described in the preceding paper by Davies (1958).

METHODS

Organisms. Strains of *Pasteurella pseudotuberculosis* used for lipopolysaccharide extraction and for immunization of animals were kindly supplied by Dr E. Thal (Veterinärmedizinischen Staatsinstitut, Stockholm). These represented each of the 5 serological groups (Thal, 1954) and in accordance with the International Bacteriological Code of Nomenclature, serological groups have been lettered. Cultures have thus been designated, e.g. 14/A/S, indicating strain number/serological group/colony form. Rough strains from some serological groups were isolated by using tryptic meat agar + peptic sheep's blood. *P. pestis* strain Tjiwidej Smooth (TS) was used for production of *P. pestis* polysaccharide which does not differ in different strains of this species (Davies, 1956).

Lipopolysaccharides. The lipopolysaccharides of *Pasteurella pseudotuberculosis* used were those described by Davies (1958). These materials have somewhat similar properties to the 'endotoxins' which have been extracted from various species of the Enterobacteriaceae (e.g. Davies, Morgan & Mosimann, 1954; Davies, 1955). The 'Rough' somatic lipopolysaccharide of *P. pestis* is that described by Davies (1956). These preparations are rather poorly soluble in water and were heated at 100° for 5 min. for use in haemagglutination inhibition tests. This treatment increases the degree of dispersion to give a stable but heavily opalescent solution. For sensitization of erythrocytes lipopolysaccharides were first treated with alkali (Macpherson, Wilkinson & Swain, 1953; Neter *et al.* 1954; Landy, Trapani & Clark, 1955). Solutions at 1 mg./ml. were heated at 37° for 18 hr. in 0·02 N-NaOH and neutralized to pH 7·0 with 0·2 N-HCl.

Antisera. For the production of antisera against whole living organisms, white rats were used because they are not susceptible to *Pasteurella pseudotuberculosis* infection. Groups of 7 rats were injected weekly by the intraperitoneal route with organisms of each of the 5 serological groups and several Rough strains, suspended in 0·5 ml. saline (0·9 %, w/v). After 2 injections of organisms equivalent to 5 μg. dry wt., the dose was raised to 100 μg.; sera from rats of each group were pooled after 9 injections had been given. Antisera against the isolated lipopolysaccharides were prepared in rabbits after combining the materials with the lipoprotein component of the *Shigella dysenteriae* 'O' somatic antigen (Partridge & Morgan, 1940; Davies, 1956). For use in the haemagglutination test sera were inactivated by heating at 56° for 30 min. and absorbed twice for 1 hr. at 37° with 10 % (v/v) of washed packed group 'O' Rh negative human erythrocytes.

Haemagglutination test. Human group 'O' Rh negative blood (citrated) was stored at 2° and replaced by a fresh stock after 30 days. Before use the erythrocytes were washed 3 times with saline and resuspended in saline at 2·5 % (v/v). In order to sensitize erythrocytes, equal volumes of this suspension and a range of serial 1/2 dilutions of alkali-treated lipopolysaccharide dissolved in isotonic phosphate buffered saline (pH 7·2) were mixed and incubated at 37° for 1 hr. These sensitized erythrocytes were washed 3 times with 10 vol. of a 1/250 dilution of normal rabbit serum in saline to remove excess lipopolysaccharide and resuspended at 0·5 % (v/v). Serial 1/2 dilutions of antiserum were prepared in 0·2 ml. volumes using a 1/100 dilution of normal rabbit serum in saline as diluent; an equal vol. of the 0·5 % sensitized erythrocyte suspension was added to each dilution of antiserum. The tubes were shaken, incubated at 37° for 1 hr. and allowed to stand at 2° overnight. Unsensitized erythrocytes for use in control tests were prepared in a similar way, but substituting buffered saline for the dilution of alkali-treated lipopolysaccharide. Controls were set up in each test, namely serum dilutions with unsensitized erythrocytes and sensitized erythrocytes with the serum diluent; when any agglutination was detected in control tubes the test was discarded. Agglutination was read microscopically, the greatest dilution at which agglutination could be detected was taken as the end-point. The sensitizing activity is recorded as the least concentration (in μg./ml.) of lipopolysaccharide which when added to the 2·5 % erythrocyte suspension gave agglutination to the maximum serum dilution. The reciprocal of this serum dilution will be referred to as the haemagglutination titre.

Haemagglutination inhibition test. This test was carried out in a similar way to that described by Morgan & van Heyningen (1944) for measurement of the serological activity of blood group substances. To serial 1/2 dilutions of heated homologous and heterologous lipopolysaccharides in saline (0·2 ml. volumes) an equal volume of serum was added suitably diluted in a 1/100 dilution of normal rabbit serum in saline to contain 3–4 haemagglutinating doses, as determined from the haemagglutination titre of the serum with the homologous lipopolysaccharide. The tubes were shaken and incubated for 1 hr. at 37°; 0·2 ml. of a 0·5 % (v/v) suspension of erythrocytes was then added to each tube, these cells having been sensitized with the homologous lipopolysaccharide at twice the concentration corresponding to its sensitizing activity. After shaking, the tubes were reincubated for 1 hr. at 37° and left at 2° overnight. Controls were: (*a*) serum with sensitized erythrocytes, which consistently gave complete agglutination; (*b*) initial dilutions of each lipopolysaccharide and serum with unsensitized erythrocytes. When agglutination occurred in control (*b*) the test was discarded. Agglutination was read microscopically and the first tube showing detectable agglutination was taken as the end-point; the dilution (inhibition titre) corresponding to this tube in the homologous system was taken to represent an activity of 100 %. The inhibiting activities of heterologous lipopolysaccharides tested have been expressed as a percentage of this activity (i.e. inhibition titre heterologous polysaccharide/inhibition titre homologous polysaccharide × 100). Differences between end-points greater than 7 tubes have been taken to indicate lack of cross-reactivity, since this would correspond to less than 1 % of the activity of the homologous standard where twofold geometrical dilutions were employed. One tube differences between end-points have been taken as significant only when the same result has been obtained at least twice.

RESULTS

Sensitizing activities of lipopolysaccharides

When representative 'Smooth' and 'Rough' *Pasteurella pseudotuberculosis* lipopolysaccharides (25/E/S and 32/D/R) were tested without alkali treatment for their ability to sensitize erythrocytes for haemagglutination, only at the highest concentration tested (500 µg./ml.) was significant (but not maximal) sensitization obtained. The *P. pestis* lipopolysaccharide, unlike any others which have been investigated, was, however, fully active (sensitizing activity 3 µg./ml.) without any alkali treatment.

Table 1. *The titration of the sensitizing activity of* Pasteurella pseudotuberculosis 32/*D*/*R lipopolysaccharide*

Lipopolysaccharide (µg./ml.)*	Serum dilution (reciprocal)							
	5	10	20	40	80	160	320	640
	Degree of agglutination†							
25	4	4	4	4	4	3	1	0
12·5	4	4	4	4	4	2	1	0
6	4	4	4	4	2	2	1	0
3	4	4	4	3	2	0	0	0
1·5	2	0	0	0	0	0	0	0

* Concentration of solution added to an equal volume of a 2·5 % erythrocyte suspension.

† Degree of agglutination: 4, complete; 3, few free cells; 2, half of cells clumped; 1, most cells free; 0, no reaction.

The sensitizing activity of each of the lipopolysaccharides after alkali treatment has been determined. These results are not given in full, but a typical test on one of the preparations (32/D/R) is shown in Table 1. From this test the sensitizing activity is taken to be 6 μg./ml., below this concentration the haemagglutination titre of the serum falls off sharply, above this concentration there is no significant increase in serum titre.

The sensitizing activities determined in this way for the series of lipopolysaccharides studied are shown in Table 2. They vary between 50 and 3 μg./ml. These figures are independent of the particular serum being used; the haemagglutination titres obtained in this series using the homologous rat antisera are also shown in this table.

Table 2. *Sensitizing activities of alkali-treated lipopolysaccharides*

Lipopolysaccharide	Sensitizing activity (μg./ml.)*	Serum haemagglutination titre†
Pasteurella pseudotuberculosis		
14/A/S	50	160
16/B/S	25	320
16/B/R	25	160
27/C/S	25	320
43/C/R	50	80
32/D/S	12·5	320
32/D/R	6	320
25/E/S	6	160
Pasteurella pestis	3	1280

* Least concentration of solution added to an equal volume of a 2·5 % erythrocyte suspension necessary to give agglutination up to maximum serum titre.

† Reciprocal of maximum serum titre.

Determination of serological specificities

The serological specificities of the *Pasteurella pseudotuberculosis* and *P. pestis* lipopolysaccharides were examined by using the haemagglutination inhibition test. For each lipopolysaccharide determinations were made of the inhibition titres as previously defined, using sera produced against each group of *P. pseudotuberculosis* and against *P. pestis*. An example of one of the 9 tests is shown in Table 3, where all the *P. pseudotuberculosis* 'Smooth' and 'Rough' lipopolysaccharides are tested for their capacity to inhibit the *P. pestis* system. In this and the following table 0 % serological activity is recorded when the inhibition titres of the homologous and heterologous lipopolysaccharides differ by 7 or more tubes. The data in Table 3 is summarized in the last line of Table 4. The data shown in Table 4, lines 1 to 5, indicate that the materials which were isolated from representatives of each 'Smooth' *P. pseudotuberculosis* group are, indeed, group specific antigens showing no overlap of specificity. In lines 6–9 (Table 4) it can be seen further that only one of the 'Smooth' group specific lipopolysaccharides show any significant inhibition of the 'Rough' *P. pseudotuberculosis* or *P. pestis* systems. In the latter part of Table 4 figures show that the lipopolysaccharides isolated from 'Rough' strains originally derived from groups B, C and D cultures and from *P. pestis* show different

Table 3. *Inhibition by Pasteurella lipopolysaccharides of the agglutination by* Pasteurella pestis *antiserum of erythrocytes sensitized with* P. pestis *lipopolysaccharide*

Antiserum against *P. pestis* was prepared in rabbits by immunization with living organisms of an avirulent strain (TS), and was used at a dilution of 1/75 (3–4 haemagglutinating doses). Degrees of agglutination as in Table 1.

Inhibiting lipopolysaccharide	Lipopolysaccharide dilution (reciprocal × 10^3)											Serological activity (%)
	2	4	8	16	32	64	128	256	512	1024	2048	
	Degree of agglutination											
P. pestis	0	0	0	0	0	0	0	0	0	0	3	100
P. pseudotuberculosis												
14/A/S	1	2	2	3	4	4	4	4	4	4	4	0
16/B/S	0	0	1	1	2	3	3	4	4	4	4	0
16/B/R	0	1	2	3	4	4	4	4	4	4	4	0
27/C/S	0	0	2	3	3	3	3	4	4	4	4	0
43/C/R	0	0	0	0	0	0	0	0	0	3	4	50
32/D/S	0	0	1	2	2	3	3	4	4	4	4	0
32/D/R	0	0	0	0	0	0	0	0	2	3	4	25
25/E/S	2	2	2	2	2	4	4	4	4	4	4	0

Table 4. *The specificities of lipopolysaccharides isolated from* Pasteurella pestis *and 'Smooth' and 'Rough' strains of* P. pseudotuberculosis

Sensitizing lipopolysaccharide	Dilution of* homologous antiserum	Inhibiting lipopolysaccharide (% serological activity)								
		14/A/S	16/B/S	16/B/R	27/C/S	43/C/R	32/D/S	32/D/R	25/E/S	*P. pestis*
14/A/S	1/20	100	0	0	0	0	0	0	0	0
16/B/S	1/20	0	100	0	0	0	0	0	0	0
27/C/S	1/20	0	0	0	100	0	0	0	0	0
32/D/S	1/15	0	0	0	0	0	100	0	0	0
25/E/S	1/15	0	0	0	0	0	0	0	100	0
16/B/R	1/15	0	0	100	0	12	0	100	0	2
43/C/R	1/5	0	0	0	0	100	6	100	0	100
32/D/R	1/30	0	0	3	0	50	0	100	0	3
P. pestis	1/75	0	0	0	0	50	0	25	0	100

* Dilution to give 3–4 haemagglutinating doses.

degrees of cross-reactivity but nevertheless possess closely related specificities. Thus, for example, the 16/B/R and *P. pestis* systems only interact very weakly, but the intermediate 32/D/R lipopolysaccharide inhibits both the 16/B/R and *P. pestis* systems.

Examination of Pasteurella pseudotuberculosis *antisera for antibodies related to the 'Rough' Pasteurella somatic antigens*

Antisera prepared in rats by immunization with living virulent *Pasteurella pseudotuberculosis* and in rabbits by immunization with living avirulent *P. pestis* were examined by the haemagglutination test for their ability to react with erythrocytes sensitized with *P. pseudotuberculosis* 'Rough' and *P. pestis* lipopolysaccharides. The sensitized erythrocytes were added to 1/2 serial dilutions of each serum, and the haemagglutination titres of the 8 'S' and 'R' antisera are shown in Table 5. These results show that antisera against the 'Smooth' *P. pseudotuberculosis* strains (except 16/B/S) contain no detectable antibody for the 'Rough' lipopolysaccharides 16/B/R and *P. pestis* and only at relatively high serum concentrations ($\nless 1/5$) could antibody be detected against 32/D/R sensitized erythrocytes. In the case of 16/B/S some agglutination ($\ngtr 1/10$) was observed with each of the 'Rough' lipopolysaccharide sensitized erythrocytes. Results obtained using 43/C/R antiserum did not differ significantly from those recorded in Table 5 for *P. pestis* antiserum. Sera against the 'Rough' *P. pseudotuberculosis* organisms (16/B/R and 32/D/R) and *P. pestis* contain antibodies which possess closely related but not identical specificities, a result which agrees with that obtained in the inhibition tests.

Table 5. *Examination of antisera to* Pasteurella pseudotuberculosis *for the presence of antibodies to 'Rough' Pasteurella lipopolysaccharides*

Sensitizing lipopolysaccharide	Antiserum							
	14/A/S	16/B/S	16/B/R	27/C/S	32/D/S	32/D/R	25/E/S	*P. pestis*
	Haemagglutination titre							
16/B/R	0*	5	80	0	0	320	0	80
32/D/R	2·5	10	80	2·5	5	320	0	640
P. pestis	0	2·5	0	0	0	160	0	1280

* Zero titre signifies no detectable agglutination at the greatest serum concentration used (1/2·5).

When antisera produced in rabbits by immunization with isolated 'Smooth' and 'Rough' *Pasteurella pseudotuberculosis* lipopolysaccharides (after combination with 'Shiga' lipoprotein) were examined in the same way, agglutination of *P. pestis* sensitized erythrocytes was obtained with both 'anti-Rough' and 'anti-Smooth' sera at dilutions down to 1/40, a result which agrees with the cross reactivity found with the same sera when precipitin tests were used (Davies, 1958).

DISCUSSION

To obtain full sensitization of erythrocytes with *Pasteurella pseudotuberculosis* lipopolysaccharides it has been necessary first to treat the materials with dilute alkali. It has been suggested (Landy *et al.* 1955) that the presence of a substantial lipid component in some of these lipopolysaccharides prevents adsorption to the erythrocyte surface and that alkali treatment affects the lipid in some way to overcome this inhibition. This is difficult to reconcile with the presence in the *P. pestis* product of *c.* 50% lipid (Davies, 1956), since this material requires no alkali treatment to elicit its full sensitizing activity, although alkaline conditions were carefully avoided during extraction and purification. The extracellular slime polysaccharide of *Klebsiella aerogenes* requires alkali treatment before it will adsorb to erythrocytes (Macpherson *et al.* 1953), but this substance contains no detectable lipid (D. A. L. Davies, unpublished observations). This is equally difficult to reconcile with the conclusions of Vogel (1957), who considered that lipid is essential for erythrocyte sensitization.

The results of haemagglutination inhibition tests show that the purified lipopolysaccharides isolated from representatives of the 5 *Pasteurella pseudotuberculosis* serological groups (Davies, 1958) are the group-specific 'Smooth' somatic antigens detected by previous workers using various serological methods. No cross-reaction was detected among the products of 'Smooth' strains using sera prepared by immunization with living *P. pseudotuberculosis* organisms.

By the phenol extraction method (Westphal *et al.* 1952) it is possible to obtain lipopolysaccharides from both 'Smooth' and 'Rough' cultures (Westphal & Lüderitz, 1954; Davies, 1957*a*). If a 'Rough' somatic antigen were present beneath the 'Smooth' somatic antigen in 'Smooth' forms, both 'Smooth' and 'Rough' components would be expected in the extracts of 'Smooth' strains. No evidence has been obtained to substantiate this classical view of S → R loss variation. The 'Smooth' *Pasteurella pseudotuberculosis* products have shown no significant neutralization of antibody against 'Rough' *P. pseudotuberculosis* strains or *P. pestis*, and antisera against *P. pseudotuberculosis* 'Smooth' strains failed to give significant agglutination of erythrocytes sensitized with 'Rough' Pasteurella lipopolysaccharides. Sera prepared by immunization of rabbits with the isolated 'Smooth' lipopolysaccharides, however, agglutinated red cells sensitized with *P. pestis* lipopolysaccharide. This is not easily interpreted but it seems possible, in view of the fact that in composition the 'Smooth' materials contain the components of the 'Rough' materials, with certain additional sugars, that 'Rough' specificity might be associated with the basic chain structure (glucosamine and aldoheptose) and 'Smooth' specificity associated with additional side chains (deoxy sugars in particular). If this is true it would be necessary to postulate further that in the intact organism, but not in the isolated product, the basic chain structure is not exposed for antibody induction. That the S → R variation is not the loss of an antigen in the sense of a discrete molecular entity, but reflects a change of polysaccharide structure, including the loss of deoxysugars, has been more clearly demonstrated in *Shigella dysenteriae* (Davies, 1957*a*, *b*).

In *Pasteurella pseudotuberculosis* the change from the virulent smooth colony form to an avirulent rough state involves more than a change in the heat-stable somatic antigen. A protein antigen is present which also appears to contribute to smoothness of colonial form; only when this antigen and the polysaccharide 'Smooth' somatic antigen are both absent is colonial form frankly rough (Crumpton & Davies, 1957). Loss of either of these two components alone does not affect the colony appearance greatly. The 'Smooth'/'Rough' protein (antigen 4) is present also in smooth colony strains of *P. pestis* which does not possess a 'Smooth' somatic polysaccharide component (Crumpton & Davies, 1956; Davies, 1956).

Examination of lipopolysaccharides extracted from 'Rough' strains of *Pasteurella pseudotuberculosis* derived from three different serological groups (B, C and D) showed that they had lost group specificity. The specificities of the 'Rough' products and the *P. pestis* lipopolysaccharide are closely related but not identical. The 43/C/R material could not be distinguished directly from that of *P. pestis*, but 32/D/R material has a specificity related to both although it is more like that of 43/C/R than the *P. pestis* product. Thus the 32/D/R lipopolysaccharide inhibited 43/C/R antiserum as effectively as 43/C/R itself, and inhibited *P. pestis* antiserum almost as effectively as 43/C/R lipopolysaccharide. But whereas the 43/C/R material inhibited 32/D/R serum as effectively as the homologous substance, *P. pestis* lipopolysaccharide reacted poorly in this test. The specificity of material 16/B/R seemed further removed from 43/C/R and *P. pestis* than 32/D/R. Thus whereas 43/C/R and *P. pestis* materials inhibit 16/B/R antisera rather weakly, 32/D/R material is as effective as the homologous substance. This overlap of specificity was also demonstrated by the cross-reactivity of antisera to 'Rough' Pasteurella strains in the agglutination of cells sensitized with the 'Rough' Pasteurella lipopolysaccharides. In the extreme case anti 16/B/R serum showed no capacity to agglutinate red cells sensitized with *P. pestis* lipopolysaccharide.

Although the 'Rough' Pasteurella lipopolysaccharides appear very similar in chemical composition (Davies, 1956, 1958) the haemagglutination technique shows that they form a spectrum of overlapping specificity in the order *Pasteurella pseudotuberculosis* 16/B/R–32/D/R–43/C/R–*P. pestis*. These are differences which could not readily be resolved by other methods. A somewhat similar situation has been described by McCarty & Lancefield (1955) for the group A specific Streptococcus polysaccharide and products of very similar chemical composition from group A variants.

The examination of antigenic patterns in bacteria by agar diffusion precipitin methods has shown in many instances a complexity not previously recognized. This is true of Pasteurellas (Crumpton & Davies, 1956, and to be published) and the presence of antigens not known to early workers in the field may account for some of the difficulties in interpreting their results, from experiments necessarily carried out with crude extracts or autolysates. Apart from the conclusion that a 'Rough' somatic antigen does not underlie the 'Smooth' somatic antigen in 'Smooth' *Pasteurella pseudotuberculosis*, and the observation that the 'Rough' products are not identical in specificity, the general picture from earlier findings agrees for the most part with the results now obtained by using purified bacterial products.

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Nutritional Studies of Cysteineless Mutants of *Salmonella typhimurium*

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SUMMARY: Forty-three separately isolated cyst(e)ineless mutants of *Salmonella typhimurium* were investigated biochemically. Six main phenotypes were identified, accommodating thirty-nine of the mutants; the remaining four mutants were of intermediate phenotype. The phenotypes are suggested to result from a series of five metabolic blocks in a pathway of cyst(e)ine synthesis in which sulphate can be reduced by two alternative routes, in both of which most of the intermediates are inorganic.

In this paper the expression cyst(e)ine is used to represent cysteine or cystine. The pathway of cyst(e)ine biosynthesis has previously been studied in a variety of micro-organisms including *Escherichia coli* (Lampen, Roepke & Jones, 1947; Roberts *et al.* 1955; Simonds 1948); *Neurospora crassa* (Phinney, 1948; Horowitz, 1950, 1955); *Aspergillus nidulans* (Hockenhull, 1949; Shepherd, 1956); *A. niger* (Steinberg, 1941). In most of these studies, the data are consistent with an inorganic pathway of sulphate reduction through sulphite and thiosulphate, and only after passing through these inorganic intermediates is the reduced sulphur atom combined with a carbon chain to form cyst(e)ine. The carbon chain has been suggested by Roberts *et al.* (1955) to be provided by serine, which was also indicated by the data of Hockenhull (1949). The irreversibility of the cyst(e)ine-methionine pathway in *Escherichia coli* was apparent from the data of both Simmonds (1948) and Roberts *et al.* (1955), although methionine was able to replace cysteine in the *E. coli* mutants studied by Lampen *et al.* (1947), and also in Aspergillus and Neurospora. In all cases, other inorganic sources of sulphur, wherever studied, gave analogous growth responses to either sulphate, sulphite or thiosulphate, and were thus considered to be rapidly converted to one of these compounds and not to be direct intermediates. Moreover, thiosulphate satisfied the growth of almost all mutants able to utilize cyst(e)ine. The data of Hockenhull (1949) and Horowitz (1955) showed that cysteic acid could replace sulphite. However, cysteine sulphinic acid could not replace thiosulphate, as would be expected if this were a direct intermediate, but had the same growth-promoting activity as the more oxidized cysteic acid. It was therefore suggested by these authors that the initial steps of the pathway involved only inorganic ions until the sulphur had been reduced to the state of thiosulphate. However, Shepherd (1956) isolated certain mutants of *Aspergillus nidulans* which were incapable of growth with sulphite and in some instances with thiosulphate, but which were able to utilize either cysteic acid or cysteine sulphinic acid. He therefore postulated alternative organic and inorganic pathways of sulphur reduction in which either serine, pyruvate or cysteic acid could act as alternative carbon sources.

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This paper describes the differentiation of a series of cyst(e)ineless mutants of *Salmonella typhimurium* by biochemical tests, from which some indication of the mode of cyst(e)ine biosynthesis by this organism may be obtained.

METHODS

Media

Defined medium. The minimal medium of Davis (Lederberg, 1950) was used, supplemented as indicated with various sulphur sources, at a concentration of 10 μg. S/ml. (unless otherwise specified), or with amino acids (20 μg./ml.). It contained 240 μg. S/ml. as sulphate.

Nutrient agar (Difco) was used when a complete medium was required.

Sulphur-free (*S-free*) *medium*, used when a medium deficient in all sulphur sources was required, was made up as tris (2-amino-2-hydroxymethylpropane-1:3 diol) glucose medium (Hershey, 1955), with the omission of sulphate.

Enriched minimal agar, used in syntrophism experiments, consisted of minimal agar containing 0·01 % nutrient broth powder (Demerec & Cahn, 1953).

Organisms

The cyst(e)ineless mutants used in this study were auxotrophs derived from *Salmonella typhimurium*, strains LT-2 and LT-7 (Zinder & Lederberg, 1952), isolated by various members of the Department of Genetics, Carnegie Institution of Washington, and made available by Dr M. Demerec.

The wild-type strains grew well on the minimal medium in which sulphate was the sole sulphur source. A series of auxotrophic mutants were isolated from both u.v.-irradiated and non-irradiated cultures of the wild-type strains by penicillin screening (Lederberg, 1950). From the auxotrophs, detected by their ability to grow on nutrient agar but not minimal agar, the cyst(e)ineless mutants were identified by their growth when L-cysteine or L-cystine was added to the minimal medium. They were designated as described by Demerec (1956) by the prefix *cys*, followed by a figure denoting the sequence of isolation; e.g. *cys-1*. In some instances, previously isolated auxotrophic strains including tryptophanless (*try*) and prolineless (*pro*) mutants were used as parental strains for the isolation of double auxotrophs. The derivation of the mutants are listed in Table 1.

Nutritional requirements of mutant strains were established auxanographically; 0·1 ml. samples of an overnight broth culture diluted 10^{-3} were spread over the surface of minimal agar plates. The agar was then removed at several places by a sterile cork-borer and drops of sterile solutions of the supplements placed into the holes. After incubation for 40 hr. at 37°, the presence or absence of haloes of growth around the holes was noted. Alternatively, the strains were spread, after appropriate dilutions, on minimal agar plates supplemented with the growth factor. After incubation at 37° for 18 hr., the plates were examined for the presence or absence of colonies of *Salmonella typhimurium.*

Table 1. *Sources of stocks of cyst(e)ineless mutants of* Salmonella typhimurium

U.v.-irradiated*		Non-irradiated	
Source	Mutants	Source	Mutants
LT-2 (wild-type)	*cys-1, -2, -3, -4, -5, -6, -7, -8, -10, -11, -12, -13, -14, -15, -16, -17, -18, -20, -21, -22, -23, -24*	LT-2 (wild-type)	*cys-36, -37, -38*
		LT-2 *try-8*	*cys-40, -41, -42, -43, -44, -50, -51*
LT-2 *pro-6*	*cys-25, -27, -28, -29*	LT-2 *try-2*	*cys-45, -46*
LT-7 (wild-type)	*cys-30, -32*	LT-2 *try-7*	*cys-47, -48, -49*

* The designation 'U.v.-irradiated' indicates that the mutants listed were obtained from cultures which had been exposed to u.v. radiation. However, since auxotrophic mutants from such cultures were isolated with no greater frequency than from non-irradiated cultures, it is likely that the mutants from both sources are largely 'spontaneous' in origin.

Syntrophism between strains was tested in two ways:

(*a*) By the parallel-streak method between pairs of mutants. Overnight broth cultures of the two mutants were diluted and streaks were made in parallel about 0·5 cm. apart on enriched minimal agar. After incubation for 5 days at 37°, the growth was then compared with single-streak controls.

(*b*) By auxanograph testing of culture filtrates and disrupted organisms. Overnight broth cultures were washed, re-inoculated into liquid minimal medium containing 4 μg. L-cysteine/ml. to give about 5×10^8 bacteria/ml., and aerated for 18 hr. at 37°. By this method the required amino acid (cyst(e)ine) was exhausted from medium and organisms; no significant reversion to prototrophy occurred at this high density of inoculation. The organisms were then centrifuged down, washed, concentrated to 2×10^{10}/ml. in minimal medium, and 10 ml. samples disrupted for 2 min. in a 'Raytheon' sonic oscillator, after which time the viable count had fallen to 10^7/ml. The supernatant fluids from the cultures in limiting cysteine-supplemented medium and the complete disruption products were then tested auxanographically for their ability to support the growth of other mutants.

Growth curves were plotted from cultures aerated at 37° in S-free media with various supplements, inoculated with about 2×10^8 washed organisms/ml. Growth was measured as optical density by means of a Klett-Summerson photoelectric colorimeter which was calibrated with a culture of the wild-type strain LT-2 in the late logarithmic phase of growth. Optical densities of serial twofold dilutions of this culture were plotted against the corresponding numbers of viable organisms/ml. obtained by counts of colonies appearing on plates of nutrient agar spread with appropriate dilutions of the culture and incubated at 37° for 18 hr. The optical density varied linearly with the number of organisms/ml. between the values of 5×10^7 and 2×10^9, organisms/ml.

Isotopic competition studies used carrier-free ^{35}S-sulphate (Oak Ridge National Laboratory; kindly supplied by Dr A. D. Hershey).

RESULTS

Growth of mutant strains on solid media

The cyst(e)ineless mutants were first tested for ability to utilize sulphate, sulphite, thiosulphate or cysteine. All strains were unable to utilize sulphate but responded to added cysteine. However, when broth cultures of certain mutants were plated on minimal agar (*c.* 10^8 organisms/plate) in which sulphate was the sole sulphur source, at least several dozen prototrophic colonies were produced. This high frequency of prototroph production made nutritional studies difficult and no further investigations were made with these strains (*cys-9*, *-19*, *-26*, *-31*, *-35* and *-39*). The growth responses shown in Table 2 (lines 3 and 4) indicate that they can be separated into four classes exhibiting growth on:

Table 2. *Growth of cyst(e)ineless mutants of* Salmonella typhimurium *in the presence of various sulphur sources*

All sulphur sources provided to give 10 µg. S/ml. (except 'thiosulphate (*a*)' provided at 1 mg. S/ml.) incorporated into minimal agar. Plates were incubated for 18 hr. at 37° after inoculation.

	Phenotype				
	cysA	*cysB*	*cysC*	*cysD*	*cysE*
	Growth response				
Sulphur source					
Cysteine	+++	+++	+++	+++	+++
Sulphate (SO_4'')	−	−	−	−	−
Sulphite (SO_3'')	++	−	+++	−	−
Thiosulphate (S_2O_3'')	−	−	+++	+++	−
Thiosulphate (S_2O_3'') (*a*)	+++	+++	+++	+++	−
Lanthionine	+++	+++	+++	+++	+++
Cysteic acid	−	−	−	−	−
Dithionate (S_2O_6'')	−	−	−	−	−
Cysteine sulphinic acid	++	−	+++	−	−
Metabisulphite (S_2O_5'')	++	−	+++	−	−
Sulphur (S)	+	−	+	+	−
Sulphide (S″)	+	−	+	+	−
Dithionite (S_2O_4'')	++	−	+++	++	−

+++ = growth equivalent to that with cysteine supplement; ++ = growth slightly less than that with cysteine supplement; + = growth greatly decreased compared to that with cysteine supplement; − = no apparent growth.

(*a*) sulphite only, *cys-1*, *-3*, *-5*, *-13*, *-20*, *-21*, *-22* and *-32* (designated *cysA* mutants);

(*b*) on neither salt, *cys-2*, *-4*, *-6*, *-8*, *-10*, *-11*, *-12*, *-14*, *-15*, *-16*, *-17*, *-18*, *-24*, *-25*, *-27*, *-30*, *-40*, *-41* and *-45* (designated *cysB* or *cysE* mutants);

(*c*) sulphite or thiosulphate, *cys-7*, *-38*, *-43* and *-47* (designated *cysC* mutants); or

(*d*) thiosulphate only, *cys-23*, *-28*, *-29*, *-36*, *-37*, *-42*, *-44*, *-46*, *-48*, *-49*, *-50* and *-51* (designated *cysD* mutants).

In subsequent experiments the mutants were streaked on minimal agar + different concentrations of sulphite or thiosulphate at 20, 50, 100, 200, 500 and 1000 μg. S/ml. The response to all concentrations of sulphite up to 500 μg./ml. was the same as that at 10 μg./ml., concentrations above 500 μg./ml. being inhibitory. On thiosulphate supplemented media, the response at all concentrations was identical to that already reported at 10 μg./ml., except at the 1 mg./ml. level, at which concentration in addition to those mutants growing at lower concentrations, a certain number of other strains unable to utilise these lower concentrations were able to grow. These mutants, which respond to thiosulphate only at a concentration of 1 mg./ml., are designated *cysB* mutants (*cys-4*, *-10*, *-12*, *-14*, *-15*, *-16*, *-18*, *-24*, *-25*, *-27*, *-40*, *-41* and *-45*) and are differentiated by this criterion from those mutants which are unable to utilize thiosulphate at any concentration tested and which form the group designated *cysE* (*cys-2*, *-6*, *-8*, *-11*, *-17* and *-30*). The mutants are thus differentiated into the five phenotypes *cysA*, *B*, *C*, *D* and *E*, as shown in Table 2 by the growth responses on sulphite and thiosulphate. The growth responses were identical when the supplements were added to minimal medium (containing sulphate) or to S-free medium and, with certain exceptions discussed later, prolonged incubation up to 5 days caused no detectable change.

The ability of other sulphur sources to support growth was then investigated. Lanthionine (β-β'-thiodialanine) allowed the growth of all mutants, but neither cysteic acid nor dithionate (S_2O_6'') stimulated the growth of any mutant. Both cysteine sulphinic acid and metabisulphite (S_2O_5'') supported the growth of the same mutants as did sulphite (*cysA* and *cysC*). Elementary sulphur and sulphide (S'') gave no stimulation of some groups, and a slow and suboptimal growth with all other strains. Dithionite (S_2O_4''), however, was a satisfactory source of sulphur for all *cysA*, *cysC* and *cysD* mutants.

The examination for syntrophism by the parallel-streaking method gave positive results only after 3 days of incubation at 37°, the plates were incubated for 5 days before scoring, when it was seen that certain *cysD* mutants (designated D_1 mutants and comprising *cys-23*, *-44*, *-46*, *-48*, *-50* and *-51*) supported the growth of all mutants in the *cysA* and *cysC* groups. Other *cysD* mutants (D_2 mutants, *cys-28*, *-29*, *-36*, *-37*, *-42* and *-49*) did not feed any other mutant strain.

Auxanograph testing of disrupted cell suspensions showed that disrupted *cysA* cells fed *cysC* mutants, and that disrupted *cysD*$_1$ cells fed either *cysC* or *cysA* mutants. Furthermore, disrupted cells of *cysC*, *cysA*, *cysB* and *cysE* mutants fed *cysD*$_1$ mutants. However, this latter stimulation of *cysD*$_1$ mutants was also given both by disrupted wild-type cells and by the culture filtrates of both wild-type and of these mutants. Apart from this apparent non-specific stimulation of *cysD*$_1$ mutants, the supernatant fluids had no growth-promoting activity on any mutant.

Growth of wild-type Salmonella typhimurium *in liquid culture*

Overnight broth cultures of wild-type strains LT-2 and LT-7 were washed in S-free medium and inoculated into similar medium, either unsupplemented or supplemented with a sulphur source at 10 μg. S/ml. (when stereoisomeric compounds were used, at 20 μg. S/ml.). The cultures were incubated with aeration at 37°, and the optical densities recorded (Fig. 1). The growth rates in cysteine, sulphate, sulphite or thiosulphate media were the same (mean generation time *c.* 50 min.), attaining a maximum of 3×10^9 organisms/ml. which was determined by the glucose content of the medium (2 mg./ml.). The corresponding curves obtained with dithionite, DL-lanthionine or cysteine sulphinic acid (not shown in Fig. 1) were similar. In S-free medium, the organisms grew initially at the optimal growth rate until a population corresponding approximately to a twofold to threefold increase had been obtained. There was little further growth. Analogous results have been described with *Escherichia coli* by Roberts *et al.* (1955) who showed that growth in S-free medium was associated with a utilization of the internal sulphur reserves of the organisms, mainly in the form of glutathione.

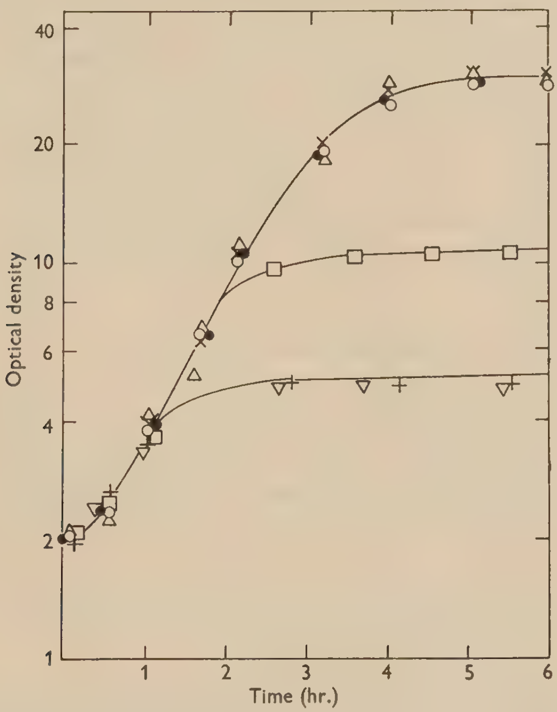

Fig. 1. Growth curves of wild-type *Salmonella typhimurium*, aerated at 37° in liquid medium with cysteine, sulphate (SO_4''), sulphite (SO_3''), thiosulphate (S_2O_3''), methionine or cysteic acid as a sole sulphur source at equiv. 10 μg. S/ml. medium. Each unit of optical density corresponds to about 10^8 bacteria/ml. ○, Cysteine; ×, SO_4''; △, SO_3''; ●, S_2O_3''; +, S-free; □, methionine; ▽, cysteic acid.

When L-methionine was the sole sulphur source, the organisms grew for a period about twice that obtained in S-free medium, after which time the growth virtually ceased. This suggests that methionine cannot act as a sole sulphur source for Salmonellas, the increase in growth over that observed in

S-free medium being due only to sparing of the internal sulphur reserves. Roberts *et al.* (1955) showed a similar phenomenon in *Escherichia coli*, and demonstrated that during growth 50% of the sulphur uptake was converted into methionine-containing protein, which would be consistent with these observations. When cysteic acid was the sole sulphur source, there was no growth beyond that obtained in S-free medium.

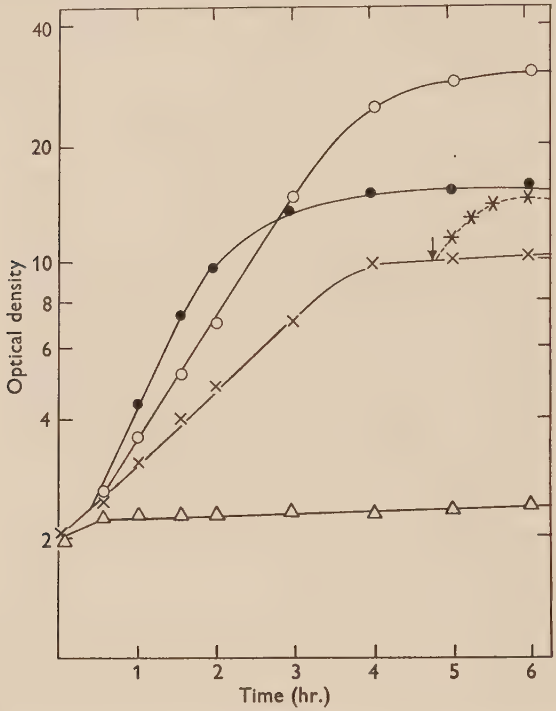

Fig. 2. Growth curves of cyst(e)ineless mutants of *Salmonella typhimurium* aerated at 37° in liquid medium with cysteine, sulphate (SO_4''), sulphite (SO_3''), thiosulphate (S_2O_3''), cysteine sulphinic acid (CSA), cysteic acid (CA), or dithionite (S_2O_4'') as a sole source of sulphur. —O—O— represents growth with cysteine (*cysA, B, C, D, E*); S_2O_3'' (*cysC, cysD*); SO_3'', S_2O_4'' (*cysC*). —×—×— represents growth with SO_3'' (*cysA*); or with S_2O_4'' (*cysA* and *cysD*). —△—△— represents growth in either S-free medium or with SO_4'' (*cysA, B, C, D, E*); SO_3'' or CSA (*cysB, D, E*); S_2O_3'' (*cysA, B, E*); and S_2O_4'' (*cysB, E*), and —●—●— represents growth of all mutants in broth. All sulphur sources provided at 10 μg. S/ml. The interrupted line shows the effect of addition at time marked by arrow of a further 10 μg. S/ml. of either SO_3'' or S_2O_4'' to the medium. Each unit of optical density corresponds to *c.* 10^8 bacteria/ml.

Growth of mutant strains in liquid culture

The growth of the cyst(e)ineless mutants in cysteine-supplemented liquid media was, in general, rather less rapid than that of the wild-type strain (Fig. 2), giving a mean generation time of about 65 min., eventually attaining the same maximum of 3×10^9 organisms/ml. In S-free medium or in sulphate medium no mutant of any group was able to grow to any significant extent. The growth of *cysC* strains in media supplemented with sulphite, thiosulphate, dithionite or cysteine sulphinic acid and that of *cysD* strains in thiosulphate-supplemented medium showed the same type of growth response as with the cysteine supplement. However, *cysA* strains in sulphite or cysteine sulphinic acid-supplemented media, and *cysA* and *cysD* strains in dithionite-supplemented medium, grew more slowly (mean generation time about 100 min.) and attained a lower maximum of *c.* 10^9 organisms/ml. No growth of *cysA* strains was observed in thiosulphate-supplemented medium, nor of *cysD* strains in sulphite or cysteine sulphinic acid-supplemented media. *CysB* and *cysE* strains could not grow on any of these sulphur sources except cysteine, in which case the growth response was similar to that given by other mutants with this sulphur source. All the mutant strains at first grew more rapidly in broth (mean generation time 35–40 min.) than in cysteine-supplemented defined medium, but there was little growth beyond 10^9 organisms/ml. Further growth to the normal (3×10^9) yield could be achieved by the addition of cysteine (5μg./ml.). Since this apparent deficiency of cyst(e)ine in broth would tend to select any reversions to wild type, cyst(e)ineless mutants were grown in broth fortified with cysteine (20μg./ml.).

Isotopic competition experiments

Wild-type organisms, after overnight growth in broth at 37° with aeration, were washed twice and inoculated into S-free medium + ^{35}S-sulphate at $0{\cdot}05\mu$c/ml. + ^{32}S-sulphate at 3μg. S/ml. to give 10^8 organisms/ml. Methionine, cysteine, sulphite, dithionite, cysteic acid or thiosulphate (all at 3μg. S/ml.) were added individually to parallel cultures. All cultures were incubated at 37° with aeration. Samples were removed at intervals, optical densities recorded and radioactivity assays made of the washed organisms. At all times the uptake of isotope by the control culture (containing ^{32}S-sulphate) was directly proportional to the amount of growth as measured by the optical density. Table 3 shows the relative radioactive uptakes of cultures containing other sulphur sources after 2·5 hr. growth, and indicates that cysteine,

Table 3. *Suppression of* ^{35}S-*sulphate uptake by* Salmonella typhimurium-LT2 *in the presence of non-radioactive competitors*

Non-radioactive supplement (*a*)	Uptake of radiosulphate (% of control) (*b*)	(*c*)
None (control)	100	100
L-Cysteine	1	1
Thiosulphate (S_2O_3'')	2	3
Dithionite (S_2O_4'')	17	60
Sulphite (SO_3'')	37	—
DL-Methionine	40	—
Cysteic acid	100	—

(*a*) Sulphur concentration of non-radioactive compound, 3μg./ml. medium.
(*b*) Total sulphate in each culture, 3μg./ml. medium.
(*c*) Total sulphate in each culture, 30μg./ml. medium.

thiosulphate and dithionite were preferred sulphur sources, in that sulphate uptake was decreased in the presence of equimolar concentrations of these compounds. Methionine or sulphite decreased the uptake to a lesser extent, and cysteic acid had little effect. The results of similar experiments using sulphate at 30 μg./ml. and 0·5 μc/ml. and certain competitors at 3 μg./ml. are also shown in Table 3.

Biochemical heterogeneity within cysB group

From Table 2 it will be seen that the group of mutants designated *cysB* can be defined nutritionally by their inability to give detectable growth on low (10 μg. S/ml.) concentrations of either sulphite or thiosulphate, although they utilize thiosulphate at higher (1 mg. S/ml.) concentrations after incubation for 18 hr.

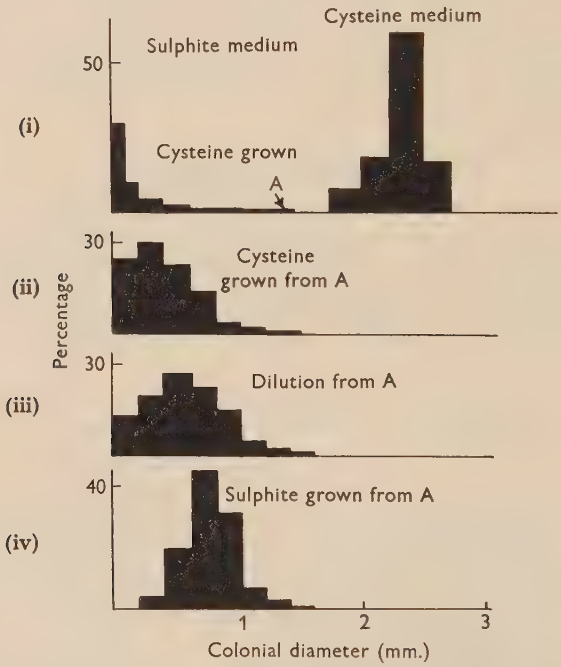

Fig. 3. Distribution of colony sizes obtained from a culture of *cysB-18* plated on minimal medium containing sulphite after the following treatments:

(i) Growth in cysteine-supplemented minimal medium (cysteine grown). Selection of a colony (A) after above treatment having a maximal size on sulphite medium (1–1·25 mm.) and replating on minimal sulphite medium after:

(ii) Growth in cysteine-supplemented liquid medium (cysteine grown from A).

(iii) Suspension in saline (dilution from 'A').

(iv) Growth on sulphite-supplemented liquid medium (sulphite grown from A).

Growth on cysteine-supplemented minimal medium was similar after all treatments and is illustrated in top right histogram.

However, when the duration of incubation on sulphite- or thiosulphate-containing (10 μg./ml.) medium was increased to 40 hr., certain of the mutants (*cysB-10*, *-18*, *-24* and *-25*) when spread at high densities, gave isolated colonies, whereas no other mutants of this group gave any perceptible growth. Since there appeared to be no concomitant growth on sulphate to suggest 'leakiness', the phenomenon was further investigated as a result of which the *cysB* mutants are subdivided into three secondary phenotypes.

First phenotype. A single clone of *cysB-18* was grown to saturation in cysteine-supplemented medium, and after centrifugation and dilution, plated on sulphite-containing minimal medium. (In all experiments, the diluted samples plated on sulphite medium were also plated on cysteine medium to give a count of viable organisms, and on sulphate medium to give a control of reversion or leakiness.) After incubation for 48 hr. at 37° *c.* 0·5 % of the organisms plated gave colonies with a diameter of about half (1·5 mm.) that formed on cysteine (3 mm.). Another 0·5 % of the organisms produced colonies which were 0·8–1·5 mm. in diameter, and a further 50 % gave colonies of diameters ranging from 50 to 800 μ. The total colonies, representing about 50 % of the number of colonies on parallel platings on cysteine medium, appeared to show a continuous variation in size from a minimum of 50 μ. to a maximum of 1·5 mm. On further incubation up to 5 days the colonial sizes increased proportionally to a maximum diameter of 3 mm., but there was little increase in the total number of colonies, and the continuous variation in size was maintained. Several of the large colonies were subcultured on sulphite medium, either immediately after dilution in saline, or after an intermediate period of growth for 18 hr. at 37° from a small inoculum (*c.* 200 organisms/ml.) in either cysteine or sulphite-supplemented liquid minimal medium. The distributions of colonial sizes on sulphite-containing medium after these treatments are summarized in Fig. 3. In no cases were colonies produced on sulphate-supplemented medium. In a limited number of experiments, mutants *cys-24* and *-25* showed characteristics similar to those of *cys-18* described above, as did all three strains on thiosulphate medium. No increase in growth was detected when any of these strains was aerated for periods up to 18 hr. at 37° in liquid minimal media + sulphite or thiosulphate.

Second phenotype. This was exhibited by *cysB-10.* Cultures grown in cysteine liquid minimal medium gave colonies of uniform size when plated on either sulphite- or thiosulphate-supplemented minimal media, although the colony diameter was only a quarter of that resulting after growth under the same conditions on cysteine medium. The number of colonies was similar on all three media. Growth curves in sulphite-supplemented medium showed no increase in lag and a slow steady growth with a mean generation time *c.* 10 hr.

Third phenotype. This is as originally described: there is no perceptible growth on sulphite or thiosulphate, except when the concentration of the latter salt is raised to 1 mg. S/ml. This phenotype is characteristic of the remaining members of the *cysB* group (*cysB-4, -12, -14, -15, -16, -27, -40, -41* and *-45*).

When *cysC* mutants were plated on the sulphite- or thiosulphate-supplemented media used in these experiments, colonies of the same number and size as those found on cysteine-supplemented medium resulted.

DISCUSSION

From the present studies the existence of a class of mutants (*cysA*) which use sulphite equiv. 10 μg. S/ml. but not thiosulphate (except at a hundred-fold concentration) makes it seem unlikely that in Salmonella, cyst(e)ine is formed from sulphate through an unbranched reductive pathway. Hockenhull (1949) suggested that sulphite may be reduced in one of several ways. The simplest route is its direct reduction through the metabisulphite (S_2O_5'') and dithionite (S_2O_4'') to thiosulphate, but it may alternatively be reduced to the unstable sulphoxylate (SO_2''). This latter compound may then either dimerize to form thiosulphate, or react non-enzymically with further sulphite to give dithionite, which is then spontaneously reduced by more sulphoxylate to thiosulphate.

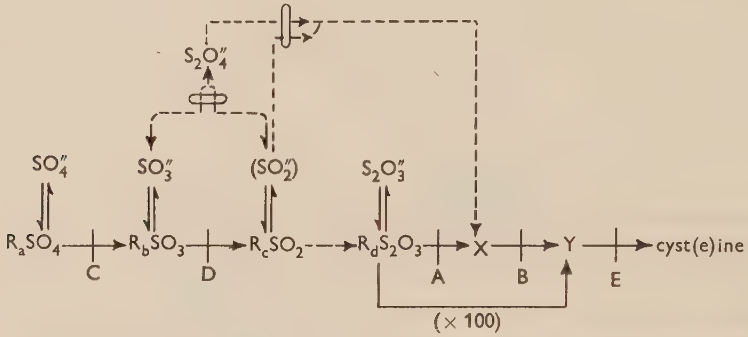

Fig. 4. Schematic diagram to represent possible pathway of sulphur metabolism in *Salmonella typhimurium*. Interrupted lines represent reactions which may occur in the absence of enzymes. R_a, R_b, etc., represent binding sites or non-diffusible organic radicals holding diffusible ions within organisms. A, B, C, D and E represent the location of metabolic blocks. Flattened circles denote that the participation of the two reactants is necessary for the reaction to proceed in the direction of the arrows.

Thus sulphoxylate, once formed, may be converted to thiosulphate in one of two ways without enzyme participation or further reduction. These interconversions, included in Fig. 4, appear to offer a satisfactory basis for summarizing the growth responses and other data obtained with the cyst(e)ineless Salmonella mutants if one assumption be made, namely, that dithionite may be reduced by sulphoxylate to give, not thiosulphate, but rather a subsequent intermediate denoted as 'X'. In this compound the sulphur atom would not have already been incorporated into the cyst(e)ine carbon chain, and since one of the sulphur atoms of the thiosulphate molecule is in the lowest reduction state, the conversion of thiosulphate to 'X' need not necessarily be by reduction. The interrupted lines in Fig. 4 denote those reactions which, according to the evidence summarized by Hockenhull, may proceed in the absence of enzymes, and which would correspond to those steps which do not appear to be under genetic control, since no mutants have been isolated with a phenotype indicating a metabolic block at these points.

According to this scheme (Fig. 4), *cysC* mutants, blocked between sulphate and sulphite, would be expected to grow at a rate equivalent to wild-type on either sulphite or thiosulphate, by utilizing either pathway. *CysD* mutants could also utilize thiosulphate by the direct pathway, but could not utilize sulphite, being unable to convert this compound to sulphoxylate. On dithionite, *cysD* mutants would be required to make use of the shunt pathway. *CysA* mutants could not utilize thiosulphate, but could utilize dithionite via the auxiliary pathway, and sulphite also by this pathway, since reduction of sulphite to sulphoxylate is possible, because of the integrity in *cysA* mutants of the step which is shown blocked at 'D' in *cysD* mutants (Fig. 4). *CysB* and *cysE* mutants would not be expected to utilize any of these inorganic salts as a source of sulphur. These metabolic potentialities are equivalent to those found by experiment and summarized in Figs. 1 and 2, and constitute the main evidence in favour of the scheme represented in Fig. 4.

The ability of *cysC* mutants to grow at the wild-type rate on dithionite may be due to its conversion to sulphite and sulphoxylate, which, according to Newton-Friend (1947), proceeds at slightly acid pH values. That *cysD* mutants do not appear to be able to utilize this pathway of dithionite metabolism may be due to the accumulation of sulphite which cannot be further reduced in this direction.

Syntrophism by certain *cysD* mutants (D_1) mutants could presumably be due to the accumulation of sulphite-containing intermediates which can be utilized by either *cysC* or *cysA* mutants, and similarly the ability of *cysA* mutants to feed *cysC* mutants might well be due to the accumulation of thiosulphate-like intermediates. This accumulation should also support the growth of *cysD* mutants, but this is unable to be demonstrated in the presence of non-specific syntrophism of these mutants with the wild-type strain and all other mutants. The inability of the other *cysD* mutants (D_2 mutants) to support growth of either *cysC* or *cysA* mutants is not readily explained from the biochemical data.

In addition, there appears to be a by-pass reaction not normally operative, but one in which the thiosulphate may be converted to the unknown intermediate 'Y' further along the pathway, allowing *cysA* and *cysB* mutants to utilize thiosulphate when this is supplied at a concentration a hundred times greater than that normally utilizable. Certain mutants of *Aspergillus nidulans* have also been found by Hockenhull (personal communication) to require concentrations of thiosulphate of 50–100 μg. S/ml. for growth.

An alternative explanation for the inability of *cysA* mutants to be accommodated within a simple unbranched pathway of sulphate reduction through sulphite and thiosulphate respectively, by reason of their inability to utilize thiosulphate (at 10 μg./ml.) might be supposed to be an impermeability of these mutants to thiosulphate, since growth can be demonstrated at the 1 mg./ml. level. However, this would imply that the purported single mutational event was capable of producing impermeability to thiosulphate, together with either an impermeability to sulphate or a metabolic block between sulphate and cyst(e)ine, since there is no growth on sulphate. The immediate uptake of radiosulphate by wild-type and mutant in the absence of growth, measured by the technique of Roberts *et al.* (1955) corresponded to between 65 and 75%

of the volume of the organism, and no difference in this uptake could be detected between *cysA* mutants and those of other mutants or of wild-type Salmonella, suggesting that there is little difference in the permeability of any of these strains to sulphate. An unbranched sequence of sulphate reduction would therefore require that all *cysA* mutants are impermeable to thiosulphate, and in addition have a metabolic block in the pathway of reduction of sulphate to cyst(e)ine.

Previously reported experiments with cysteine sulphinic acid support the concept of an inorganic pathway at least as far as thiosulphate in all organisms except *Aspergillus nidulans* (Shepherd, 1956). In this latter study, alternative inorganic and organic pathways of sulphur reduction were suggested, but the proposed scheme required one of the mutants to have simultaneous metabolic blocks in two of the pathways concerned. Moreover, in *Escherichia coli* the ability of pyruvate to supply the carbon source for cyst(e)ine synthesis, as postulated by Shepherd, was considered unlikely by Roberts *et al.* (1955) because of its inability to compete with glucose for this function, whereas under the same conditions serine was able almost entirely to suppress the contribution of carbon from glucose to cyst(e)ine.

The concept of an inorganic pathway of reduction is supported by the present studies, since no accumulation of either cysteic acid or cysteine sulphinic acid was detected in the supernatant fluid or disrupted cells of representative mutants or wild-type grown in the presence of radiosulphate and tested chromatographically by the methods of Chapeville & Fromageot (1954), even though these disrupted cell preparations were active in stimulating the growth of other mutants as detailed previously. The inability of sulphide and free sulphur to give adequate growth responses supports similar findings by Hockenhull and by Steinberg, who suggested that these compounds were unlikely to be direct intermediates. The isotopic competition experiments suggest that thiosulphate in particular is a direct intermediate in view of its ability almost completely to exclude sulphate uptake on an equimolar basis. The ability of this compound to act as a competitor to sulphate was even greater in the experiments of Roberts *et al.* (1955) with *Escherichia coli.* However, it would appear that the demonstration by these same authors of the ability of sulphite to suppress sulphate uptake to a much greater extent in *E. coli* than was found in Salmonella suggests a different mechanism of participation of sulphite in the two systems. Moreover, *Salmonella typhimurium* appears to differ from previously studied organisms by its inability to utilize cysteic acid as a sulphur source.

The lack of apparent syntrophism except after 3 days of incubation at 37° with growing organisms and the presence of growth-promoting substances within the cells even after a dilution of 1/500, compared with the inactivity of the corresponding supernatant fluids suggests that these intermediates are not diffusible, and are normally liberated only after autolysis. It would appear, therefore, that if they are inorganic ions they do not exist in a free state but may be bound by some large non-diffusible organic radicals, at sites within the cell (Roberts *et al.* 1955), which are denoted as R_a, R_b etc. in Fig. 4.

The mutants *cys-10*, *-18*, *-24* and *-25*, classified provisionally as *cysB* mutants on the basis of primary nutrition in Table 2, appear on further investigation to be intermediate in phenotype to *cysB* and *cysC*, and cannot be easily accommodated within the proposed pathway.

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Investigation of the Genetics of Cysteineless Mutants of *Salmonella typhimurium* by Transduction

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SUMMARY: The genetic investigation of forty-three phenotypically classified cysteineless mutants of *Salmonella typhimurium* by means of transduction suggests that in forty-one of the mutants, strains of similar phenotype result from mutations occurring at a series of sites which form a closely linked group, presumably situated within a single gene locus. Five such loci have been identified.

Only two of these five presumptive loci are linked, and within these linked loci an unusually high proportion of mutations to auxotrophy appear to result from a chromosomal deficiency. Another locus which is linked to an array of loci controlling tryptophan biosynthesis shows biochemical heterogeneity within the locus.

The transfer of hereditary characters between strains of *Salmonella typhimurium* by means of temperate bacteriophage (transduction) was first described by Zinder & Lederberg (1952). It is believed that during the process of lytic development in one strain (donor), a proportion of the phage particles incorporate a fragment of bacterial genetic material. When such particles infect another strain (recipient), this fragment of the bacterial genome is transferred, together with the genetic material of the phage. In those recipient cells which survive the infection, the fragment may replace the homologous part of the genetic material of these cells. This latter process has been shown to be accomplished by a mechanism analogous to crossing-over (Demerec & Demerec, 1956). The incorporated fragment is apparently small, since in most instances only a single discrete character can be transduced at a time, although instances of simultaneous transfer of apparently linked characters have been reported (Stocker, Zinder & Lederberg, 1953; Demerec *et al.* 1954; Demerec & Hartman, 1956; Hartman, 1956; Yura, 1956).

The efficiency with which non-exacting (prototrophic) cells may be produced after treatment of one mutant with phage propagated on another mutant has been studied in detail among a large number of separately isolated auxotrophs of *Salmonella typhimurium* by Demerec and his co-workers. These mutants which have a growth requirement for a specific amino acid, vitamin or purine were classified phenotypically by their ability to grow on certain intermediates in the synthetic pathway of the particular growth factor, and also by cross-feeding tests between mutants responding to different precursors in the same pathway. Transduction experiments showed when two mutants have an auxotrophic requirement for different growth factors, that under standard conditions, the number of prototrophs formed is similar to that found when either mutant is infected with phage derived from the lysis of wild-type cells. However, although transductions between most pairs of the mutants of the same phenotypic group were found to give rise to prototrophs, they were produced in much smaller numbers than when wild-type cells were used as the source for the phage. This suggested that these apparently phenotypically identical mutants (manifesting the same metabolic block) were not genetically identical, but that the mutational sites involved were probably very closely linked, so that crossing-over between them, required to produce a prototroph, was infrequent, which would result in a decreased yield of prototrophs by transduction (Demerec *et al.* 1954).

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The evidence is consistent with the view that a gene locus extends over a finite region of the chromosome, the integrity at many points along this region being necessary to ensure the continuation of the particular function which is controlled by the gene in the wild-type cell. Changes occurring at any one of these different points (sites) within this region will thus give rise to mutants which will be of a similar phenotype, in that the function controlled by the gene is either deficient or modified in each mutant. The mutational sites are separable by crossing-over, which can thus give rise to the wild-type genome. The term ‘gene’ or ‘gene locus’ is hereafter used to mean that unit, defined by terms such as ‘complex locus’, which corresponds to the gene as a physiological unit of function, and which can be further subdivided by either mutation or recombination. Mutations occurring within this locus will thus produce a series of mutants which are ‘non-identical alleles’ of the same gene locus, and which resemble each other in the loss or modification of the particular function which is under the control of the gene concerned (Demerec, 1956*a*; Benzer, 1957).

In general, within one locus, the nearer the relative location of two sites, the smaller is the yield of prototrophs obtained by transduction of one mutant by phage propagated on the other (Demerec *et al.* 1954).

The investigation of a series of cysteineless mutants of *Salmonella typhimurium*, which have been previously characterized phenotypically (Clowes, 1958) is reported in this paper.

METHODS

Salmonella typhimurium, LT-2 (Zinder & Lederberg, 1952) was used as a wild-type strain.

Cysteineless mutants used in this study comprised the following strains:

(*a*) *cysA* mutants (*cys-1*, *-3*, *-5*, *-13*, *-20*, *-21*, *-22* and *-32*) in which sulphite can replace the cysteine requirement.

(*b*) *cysB* mutants (*cys-4*, *-10*, *-12*, *-14*, *-15*, *-16*, *-18*, *-24*, *-25*, *-27*, *-40*, *-41* and *-45*) in which the cysteine requirement can be replaced by high concentrations (1 mg. S/ml.) of thiosulphate.

(*c*) *cysC* mutants (*cys-7*, *-38*, *-43* and *-47*) whose cysteine requirement can be either sulphite or thiosulphate ($>10\mu$g. S/ml.).

(*d*) *cysD* mutants (*cys-23*, *-28*, *-29*, *-36*, *-37*, *-42*, *-44*, *-46*, *-48*, *-49*, *-50* and *-51*) able to replace the cysteine requirement with thiosulphate ($>10\mu$g. S/ml.).

(*e*) *cysE* mutants (*cys-2*, *-6*, *-8*, *-11*, *-17* and *-30*) whose growth requirement for cysteine cannot be replaced by any inorganic sulphur source.

Their isolation and classification is described elsewhere (Clowes, 1958).

Phage stocks used in all transductions were derived from the temperate form (H_1) of phage PLT-22 of Zinder & Lederberg (1952). The stocks were prepared by infecting logarithmic-phase cultures of the donor strain (10^8 bacteria/ml.) in nutrient broth with phage at a multiplicity (phage particles per bacterium) of 0·005. The previous host of the phage is immaterial, since transducing ability is not maintained through successive lytic propagations of the phage (Zinder, 1955). After aeration for 5 hr. at 37°, the partially lysed cultures were centrifuged at 3000–5000 *g* for 15 min., to remove unlysed bacteria and debris, and the supernatants were further centrifuged at 12,000 *g* for 1 hr. The phage was thus sedimented as a translucent pellet, which, after decantation of the supernatant, was suspended in approximately one-tenth the original volume of T2 buffer (Hershey & Chase, 1952). Residual bacteria were killed by the addition of chloroform, which was removed by aeration after 10 min. These concentrated phage preparations were then assayed, the titre at this stage being usually between 4×10^{11} and 10^{12} phage particles/ml. Stocks were maintained at 4°, at which temperature their titre was constant over several months.

Phage assays were made by the agar layer technique (Adams, 1950), using strain LT-2 as indicator.

Media used were as detailed previously (Clowes, 1958).

Transduction was effected by mixing recipient bacteria in the late logarithmic phase (10^9 cells/ml.) with phage at a multiplicity of 5 at 37°, and holding at this temperature for at least 5 min. to allow adsorption. This mixture was then spread in 0·1 ml. vol. over enriched minimal agar plates by means of a glass spreader. Controls of uninfected bacteria were plated on the same medium, as was an appropriately diluted phage suspension. In order to control variations in transduction efficiency due to apparent physiological variation in parallel cultures of the same recipient strain, every culture in each series of transductions was also treated with a standard preparation of phage which had been propagated on wild-type cells. Thus for each mutant the numbers of prototrophs produced by phages grown on other mutants could be expressed in terms of the numbers of prototrophs produced by phage propagated on wild-type cells. All transduction mixtures were plated on at least three plates, with three control plates. All recipient strains were sensitive to PLT-22. Colonies were counted after 36 hr. incubation at 37°, and where these consisted of various phenotypes (e.g. Tables 5 and 6), the numbers of the individual phenotypes were scored after replication by the velvet-pad technique (Lederberg & Lederberg, 1952) on appropriately supplemented minimal agar plates which were incubated for 6 hr. at 37°.

In most experiments, a few isolated colonies were found on the plates spread with uninfected bacteria. These colonies were stably prototrophic, and probably arose as a result of either back-mutation (reversion) or further mutation at a different locus (suppression), in some rare mutant cells. In general, these colonies occurred with a frequency of about one for every 10^8 cells plated, and in these instances, the yields of prototrophic colonies arising on plates spread with the phage + bacteria mixture were corrected for this phenomenon. In rare instances, a score or more colonies were produced, presumably due to mutation at an early stage after subculture and under these circumstances, the transduction was discarded and the experiment repeated with a fresh subculture. Since all mutants used in this study had been previously screened for stability in this direction, and unstable mutants which invariably produced prototrophs at a frequency greater than about 1 in 10^7 cells plated had been previously discarded (Clowes, 1958), a further subculture usually resulted in a low colony count on the uninfected bacteria control plates. With some mutants, no colonies were detected on minimal agar plates spread with the usual 10^8 inoculum. With certain of these strains, no reversion was noted on ten or more plates spread with 10^9 cells. These non-reverting strains were restricted to certain members of the *cysD* group (D_2 mutants) which had been previously differentiated (Clowes, 1958) from the remainder of *cysD* group (D_1 mutants) by their inability to support the growth of *cysC* mutants by syntrophy (*cysD-28*, *-29*, *-36*, *-37*, *-42* and *-49*).

In all instances, cultures of mutants infected with phage derived from the lysis of a separate subculture of the same mutant (homologous phage) gave numbers of prototrophic colonies which were similar to the corresponding uninfected controls.

In some experiments, where the numbers of colonies appearing after transduction were small, the multiplicity of the phage was increased to 25, and ten plates of both transduction mixture and controls were spread. In these instances it has been assumed that the efficiency of transduction varies linearly with multiplicities between 5 and 25 (Zinder & Lederberg, 1952), and the results are expressed as those expected at the lower multiplicity.

Abortive transduction of nutritional characters was investigated by the examination of plates spread with a transducing mixture of phage and bacteria for 'minute colonies' (Ozeki, 1956). The term 'abortive transduction' was coined by Stocker *et al.* (1953) for the 'trail' phenomenon observed in experiments involving the transduction of motility. These authors suggested that in certain instances after infection the genetic fragment introduced into the recipient cell might not replicate and was not eliminated. A similar mechanism was invoked by Ozeki (1956) to explain phenomena observed during the study of transduction of nutritional characters. In this instance, a number of 'minute' colonies were observed, in addition to the larger prototrophic colonies, on minimal plates spread with the transduction mixture. Experimental evidence was consistent with the hypothesis that each 'minute' colony contained only one cell which was capable of producing a further 'minute' colony. Unilinear inheritance of a non-replicating non-eliminated fragment carrying the dominant wild-type allele of a nutritional mutation was invoked to explain the phenomenon which was considered analogous to the 'trail' phenomenon described by Stocker *et al.* (1953). The observation of 'minute' colonies was first made in transductions using phage derived from wild-type donors, but it was later noted that phage propagated on certain mutants was also capable of eliciting 'minute' colony formation when used to infect certain other mutants. However, when the phage was derived from a mutant which was phenotypically similar to the recipient strain, so that the mutational sites of both donor and recipient strains were located within the same gene locus controlling the same function, 'minute' colonies did not develop. Conversely, phage propagated on strains whose sites of mutation were not in the same functional unit as that of the recipient strain invariably gave rise to 'minute' colonies.

The ability of two mutants to produce 'minute' colonies in this manner suggests, therefore, that the mutational sites of recipient and donor strains are situated in different functional units, and thus in different gene loci. Abortive transduction seems therefore capable of differentiating a series of linked mutational sites into separate gene loci in the absence of any biochemical test, and as such seems comparable to the test for complementarity of two mutants combined in a heterokaryon (Pontecorvo, 1952).

RESULTS

Transduction was carried out with almost all the possible combinations of mutants and in both directions within each of the phenotypic groups of mutants designated as *cysB*, *C*, *D* and *E*. With certain exceptions, therefore, each mutant of a group was treated with phage grown on every member of that group (Tables 1–3). In addition, each mutant was treated with phage from the same wild-type lysate. It will be seen that there is a large variation in the efficiency with which prototrophs were produced by different mutants when so infected. Moreover, there was an overall twofold variability in the yield of prototrophs from different subcultures of the same strain when treated with any phage. However, the relative efficiency with which a certain subculture of any one strain produced prototrophs when infected by phage propagated on a variety of donor strains, including wild-type, was fairly constant. This led to the expression of prototrophic yields as a fraction of the prototrophs produced by the same subculture of the strain when infected with wild-type phage.

In general, the yield of prototrophic colonies obtained by transduction between mutants of the same phenotypic group was lower than the corresponding yield involving wild-type donors or phenotypically dissimilar mutants. However, in the case of *cysC* and *cysD* mutants, many intragroup transductions gave rise to larger numbers of prototrophs than certain intergroup transductions. Each mutant in both groups was therefore treated with phage from every member of both groups (Table 2). Furthermore, *cys-48* and *cys-50*, classed as *cysD* mutants by phenotype, behaved in all transductions tested as though unlinked to any other mutant (including those of either the *cysC* or *cysD* group), in giving yields of prototrophs of the same order as with wild-type donors, although giving reduced yields of prototrophs in transductions between each other (Table 2).

Transduction among the *cysA* group of mutants has been previously described by Demerec (1955). In this study, in all possible crosses between *cysA-1*,

-3, *-5*, *-13*, *-20*, *-21* and *-22*, and in further studies involving *cys-32* (Demerec, unpublished) the maximum yield of prototrophs in any one cross was 44 %, that found using phage from the wild-type strain. However, the reciprocal of this particular inter *cysA* cross gave only 14 % wild-type yield, and three only of the total possible sixty-four combinations (including homologous crosses) gave yields of prototrophs greater than 10 % of the corresponding yield using the wild-type strain as donor. The results of transduction of certain of the *cysB* mutants included in Table 1 have previously been reported by Demerec, Blomstrand & Demerec (1955). From these experiments, and from those summarized in Tables 1–3 with the exception of *cys-48* and *cys-50*, the cysteineless mutants can thus be divided into four transduction groups corresponding respectively with the groups *cysA*, *cysB*, *cysE* and (*cysC*+*cysD*), previously established by the study of the nutritional requirements of the mutants (Clowes, 1958).

Table 1. *Frequency of production of prototrophic colonies in transductions between members of cysB group*

	Phages grown on													
Recipient bacteria	(*a*) Mutants													(*b*) Wild-type
	cys-4	*-10*	*-12*	*-14*	*-15*	*-16*	*-18*	*-24*	*-25*	*-27*	*-40*	*-41*	*-45*	
cys-4	0	0·5	*	*	*	*	1·8	0·9	*	*	1·2	0·8	8·3	2800
cys-10	0	0	6·9	6·2	9·6	3·6	4·1	1·6	4·1	3·7	4·0	4·4	3·2	2474
cys-12	*	1·3	0	2·0	3·9	1·6	1·7	1·2	2·0	6·7	3·0	4·3	3·0	2104
cys-14	*	1·6	2·1	0	1·8	0·2	0·2	0·5	5·0	2·2	1·4	3·7	18·0	1792
cys-15	*	8·0	10·1	11·2	0	5·4	9·2	18·3	13·3	32·5	28·5	32·1	36·3	1096
cys-16	*	1·8	1·9	0·2	3·5	0	0·2	1·0	1·8	1·1	0·5	1·4	7·7	979
cys-18	3·0	0·6	2·6	0·1	3·1	0·4	0	0·4	0·5	0·3	0·9	0·5	2·1	2964
cys-24	3·0	1·5	2·9	1·1	5·2	0·6	0·5	0	0·1	0·6	0·6	6·2	0·8	5175
cys-25	*	6·5	5·8	1·1	6·3	0·9	0·7	0·7	0	2·5	1·4	4·5	14·0	2130
cys-27	*	4·9	2·4	1·7	8·5	0·4	0·6	0·6	2·8	0	1·0	3·6	7·5	888
cys-40	7·3	11·4	1·9	0·5	5·3	0·2	0·2	0·4	0·6	0·8	0	1·0	11·0	1263
cys-41	4·2	2·4	1·3	1·1	5·7	0·6	8·0	4·9	1·4	1·4	0·5	0	1·9	1343
cys-45	3·1	4·0	0·5	1·0	23·9	2·4	0·5	1·3	0·75	1·0	1·4	1·5	0	1517
cysD-23	113	71	57	43	58	64	37	40	75	59	79	82	108	1265

(*a*) Each figure represents, for each bacterial strain, the value:

$$\frac{\text{number of prototrophic colonies obtained with phage grown on mutant}}{\text{number of prototrophic colonies obtained with phage grown on wild-type cells}} \times 100.$$

Each plate was spread with 2×10^8 cells and 10^9 phage which had been incubated together at 37° for 10 min. The figures shown are the average of at least two separate experiments, in each of which a minimum of three plates were used for each mixture.

(*b*) Figures for phage grown on wild-type represent total number of colonies appearing on three plates each spread with 2×10^8 mutant cells and 10^9 phage particles in a typical experiment.

* No crosses made.

Linkage between groups was investigated by several methods.

(1) *Yield of prototrophic colonies from transduction between representative members of different phenotypic groups.* In these experiments, Table 4 shows the yields of prototrophs to be of the same order as those observed with wild-type donor phage except between *cysC* and *cysD* mutants (cf. Table 2), where

Table 2. *Frequency of production of prototrophic colonies in transductions between members of cysC and cysD groups*

Recipient bacteria		Phages grown on																
		(*a*) Mutants																(*b*) Wild-type
		cysD$_2$						*cysD*$_1$				*cysC*				*cysD*$_1$		
		-37	*-42*	*-49*	*-28*	*-29*	*-36*	*-46*	*-44*	*-23*	*-51*	*-38*	*-7*	*-47*	*-43*	*-48*	*-50*	
cysD$_2$	*-37*	0	0	0	0	0	0	0	0	0	0	0	0	0	17	*	*	402
	-42	0	0	0	0	0	0	0	0	0	0	0	0	0	23	*	*	367
	-49	0	0	0	0	0	0	0	0	0	0	0	0	0	25	*	*	181
	-28	0	0	0	0	0	0	0	0	0	0	0	0	0	33	*	*	468
	-29	0	0	0	0	0	0	0	0	0	0	0	0	0	45	*	*	206
	-36	0	0	0	0	0	0	9·6	9·2	0·9	0	0	0	0	80	*	*	291
cysD$_1$	*-46*	0	0	0	0	0	3·5	0	53	1·5	1·8	0·1	9·5	16	135	*	*	654
	-44	0	0	0	0	0	0	21	0	10	0·2	1·2	38	10	50	*	*	1494
	-23	0	0	0	0	0	1·3	2·5	20	0	4·6	3·6	6·3	6·5	77	206	*	1695
	-51	0	0	0	0	0	0	9·5	10	8·2	0	2·4	3·8	12	157	*	*	695
cysC	*-38*	0	0	0	0	0	0	21	14	5	1·2	0	0·9	1·2	51	185	*	1680
	-7	0	0	0	0	0	0	6·1	42	10	0·5	0·1	0	2·4	115	72	*	1448
	-47	0	0	0	0	0	0	3	19	2	0·3	0·02	0·3	0	62	*	*	354
	-43	9·1	14	0·2	6·6	9·5	8·9	60	54	42	35	18	23	37	0	*	*	517
cysD$_1$	*-48*	*	*	*	*	*	*	*	*	101	*	77	85	*	*	0	0·8	161
	-50	*	*	*	*	*	*	*	*	79	*	*	127	*	*	14·3	0	790

(*a*) Each figure represents, for each bacterial strain, the value:

$$\frac{\text{number of prototrophic colonies obtained with phage grown on mutant}}{\text{number of prototrophic colonies obtained with phage grown on wild-type cells}} \times 100.$$

Each plate was spread with 2×10^8 cells and 10^9 phage which had been incubated together at 37° for 10 min. The figures shown are the average of at least two separate experiments in each of which a minimum of three plates were used for each mixture.

(*b*) Figures for phage grown on wild-type cells represent total number of colonies appearing on three plates each spread with 2×10^8 mutant cells and 10^9 phage particles, in a typical experiment.

* No crosses made.

Table 3. *Frequency of production of prototrophic colonies in transductions between members of cysE group*

Recipient bacteria	Phages grown on: (*a*) Mutants: *cys-2*	*cys-6*	*cys-8*	*cys-11*	*cys-17*	*cys-30*	(*b*) Wild-type
cys-2	0	0	1·1	0·5	2·3	0	336
cys-6	0	0	1·2	5·9	3·2	0	512
cys-8	0	0	0	0·4	0	2·6	327
cys-11	0·5	6·0	0·2	0	1·3	1·4	381
cys-17	1·0	0	0	2·9	0	0·3	278
cys-30	0	0	0	3·0	8·6	0	276
cysD-23	84	60	39	95	97	54	1011

(*a*) Each figure represents, for each bacterial strain, the value:

$$\frac{\text{number of prototrophic colonies obtained with phage grown on mutant}}{\text{number of prototrophic colonies obtained with phage grown on wild-type cells}} \times 100.$$

Each plate was spread with 2×10^8 cells and 10^9 phage which had been incubated together at 37° for 10 min. The figures shown are the average of at least two separate experiments in each of which a minimum of three plates were used for each mixture.

(*b*) Figures for phage grown on wild-type cells represent total number of colonies appearing on three plates each spread with 2×10^8 mutant cells and 10^9 phage particles, in a typical experiment.

Table 4. *Frequency of production of prototrophic colonies in transductions between cysteineless mutants of various phenotypic groups*

Recipient bacteria		Phages grown on: *cysA*		*cysB*				*cysC, cysD*			*cysE*			*cysD*
		-3	*-20*	*-4*	*-10*	*-18*	*-24*	*-7*	*-38*	*-23*	*-2*	*-11*	*-30*	*-48*
cysA	-3	0	0	157	185	287	141	47	205	155	75	114	219	55
	-20	0	0	171	247	206	120	52	62	125	97	117	90	220
cysB	*-4*	102	53	0	0·5	1·8	0·9	61	24	75	32	46	30	*
	-10	52	32	0	0	4·2	1·6	40	51	59	70	86	87	66
	-18	88	132	3	0·6	0	0·4	102	89	45	66	77	111	*
	-24	75	46	3	1·5	0·5	0	56	92·5	126	35	53	116	47
cysC	*-7*	133	148	131	68	94	129	0	0·1	10	124	102	90	72
	-38	152	147	146	105	150	133	0·9	0	5	66	81	72	185
cysD-23		59	119	113	83	91	40	6·3	3·6	0	84	95	54	206
cysE	-2	161	136	154	95	80	67	125	210	144	0	0·5	0	54
	-11	88	206	175	81	133	57	120	138	141	0·5	0	1·4	93
	-30	73	153	112	111	61	88	121	172	109	0	3	0	127
cysD-48		80	138	*	98	88	122	85	77	101	86	72	.52	0

Each figure represents, for each bacterial strain, the value:

$$\frac{\text{number of prototrophic colonies obtained with phage grown on mutant}}{\text{number of prototrophic colonies obtained with phage grown on wild-type cells}} \times 100.$$

Each plate was spread with 2×10^8 cells and 10^9 phage which had been incubated together at 37° for 10 min. The figures shown are the average of at least two separate experiments in each of which a minimum of three plates were used for each mixture.

* No crosses made.

the majority of the prototrophic yields were less than 20 % of those found using phage from wild-type cells. In contrast, *cys-48* behaved as unlinked to any mutant of groups *cysA*, *cysB*, (*cysC*, *cysD*) or *cysE*. However, since day-by-day variations as great as twofold were experienced in prototrophic yields, this method can be taken to exclude only close linkage between the mutants of different groups.

(2) *Linkage to the array of tryptophan markers* of certain *cysB* mutants was shown by Demerec & Demerec (1956); a double mutant carrying any one of several tryptophan markers and a *cysB* marker when treated with phage grown on wild-type cells was found to give a significant number of prototrophic colonies, in addition to the cysteine-dependent and tryptophan-dependent colonies resulting from transduction of one or another marker only. A number of *cys* mutants of each transduction group was tested for linkage with certain *try* markers, either in this way, or, when double mutants (*try.cys*) were unavailable by treatment of the *cys* auxotroph with phage from *try* donors. Plating on minimal medium supplemented with tryptophan and subsequent replication on unsupplemented minimal agar permitted the identification of any *try* colonies whose presence would reveal linkage of the *try* and *cys* markers (donor-phenotype selection). The results obtained indicated linkage of the *try* loci to the genetic markers of all the mutants of the *cysB* group. No such linkage was found in the tests of representatives of other *cys* groups (*cysE-2*, *-8*, *-11*, *-17*, *-30*; *cysC-43*, *-47*; *cysD-42*, *-44*, *-46*; *cys-48*, *-50*; *cysA-1*, *-21*). It would thus appear that the *try* loci and therefore the *cysB* locus is not linked to either the *cysA*, *cysC*, *cysD* or *cysE* loci, nor to *cys-48* or *-50*.

(3) *Donor-phenotype selection* between phenotypically distinct, cysteineless mutants can be accomplished when the donor cells utilize a sulphur source which does not support the growth of the recipient cells; e.g. in a cross of *cysB* (bacteria) with *cysA* (phage), if *cysA* and *cysB* groups are linked, any $cysA.cysB^+$ cells resulting from simultaneous transduction of the two markers will have the *cysA* phenotype and will produce colonies on sulphite-supplemented minimal agar, on which cells of the recipient *cysB* strain do not grow. Colonies of this type can be distinguished from the wild-type ($cysA^+.cysB^+$) colonies by their inability to grow when replicated on unsupplemented minimal agar.

The simultaneous transfer of two markers is only expected when the loci concerned are closely linked. It was experimentally feasible to apply this test in the cases shown in Table 5, which includes all possible combinations of groups except that of *cysB* and *cysE*. Donor-phenotype colonies were not detected in any of these crosses, except of those mutants (*cysD*, *cysC*) already shown to be linked by prototrophic yields. In these transductions, cells of the *cysC* phenotype were recovered with a frequency of as high as nine times that of wild-type, showing very close linkage of the *cysC* and *cysD* loci. In all other cases, all the colonies appearing on the supplemented minimal agar plates grew on unsupplemented minimal agar and were thus prototrophic (in each transduction about 1000 colonies appearing on a total of three supplemented plates were replicated). The sensitivity of the test, which is restricted by the velvet-pad replica technique, is such that linkage greater than about 2 % should have been detected.

Abortive transduction. 'Minute' colonies attributable to abortive transduction were sought for in transduction experiments involving recipient strains of each group treated with phage grown on wild-type cells or on mutants of the same or different groups. Crosses were examined in all possible combinations among *cysA-1*, *B-14*, *C-7*, *D-23* and *E-30*, between certain *cysA* mutants (*cys-1* and *-3*) and among certain *cysB* mutants (*cys-14*, *-18*, *-45*, *-10* and *-15*). These crosses included all reciprocal crosses and homologous crosses, and in addition, crosses of all mutants with phage from the lysis of the wild-type strain. *CysA*, *cysB* and *cysE* mutants gave distinct minute colonies with phage grown either on wild-type cells or on any mutant not of the same group as the recipient. They did not produce minute colonies when treated with phage grown on mutants of the same group as the recipient (though large wild-type colonies were produced). So far as was tested, therefore, the groupings of these mutants by abortive transduction confirmed the groupings by biochemical criteria. The background growth of *cysC* and *cysD* mutants make detection of minute colonies difficult, so that it was impossible to apply the test to recipient strains of either of these two groups.

Table 5. *Selection for 'donor-phenotype' between cysteineless mutants of different phenotypic groups*

Recipient bacteria	Mutant used for propagation of phage	Supplement added to minimal agar (*a*)	Percentage of transduced colonies which are prototrophic
cysA-1, -3	*cysC-7*	Thiosulphate	100
	cysD-23	Thiosulphate	100
cysB-18	*cysA-1*	Sulphite	100
	cysC-7	Thiosulphate	100
	cysD-23	Thiosulphate	100
cysD-23	*cysA-1*	Sulphite	100
	cysC-7	Sulphite	10
cysE-30	*cysA-1*	Sulphite	100
	cysC-7	Thiosulphate	100
	cysD-23	Thiosulphate	100
	cysD-48	Thiosulphate	100
cysD-48, -50	*cysA-1*	Sulphite	100
	cysC-7	Sulphite	100

(*a*) Supplements were added at a concentration of 10 μg. S/ml. In each experiment, three supplemented minimal plates were spread, each with 2×10^8 recipient cells and 10^9 phage particles. After incubation for 18 hr. at 37° the plates were replicated both to minimal agar, and to minimal agar with the same supplement, and examined for colonies after incubation for 5–6 hr. at 37°.

Mapping of cysB locus by three-point tests. By linkage experiments, the markers of all *cysB* mutants have been shown to be linked with the tryptophan loci, whereas mutants of other groups show no such linkage. The data in Table 1 confirm this close linkage of all the sites within the *cysB* group, and those of Table 4 show the absence of linkage between the markers of certain randomly chosen mutants of this group and the markers of other *cys* groups. Nutritional requirements, however, subdivide these mutants into three phenotypes: B_1 (*cysB-4, -12, -14, -15, -16, -27, -40, -41* and *-45*), in which there is no perceptible growth on either sulphite or thiosulphate; B_2 (*cysB-18, -24* and *-25*), in which the ability to utilize sulphite or thiosulphate appears to result from slow adaptation by a very small proportion (*c.* 1%) of the cells; and B_3 (*cysB-10*), in which all the cells in a culture are equally able to utilize either sulphite or thiosulphate, though at a slower rate than any mutants in *cysC* and *cysD* groups. In all these experiments, the concentration of sulphur supplied as thiosulphate was that amount found satisfactory when supplied as cysteine (10 μg. S/ml.) (Clowes, 1958).

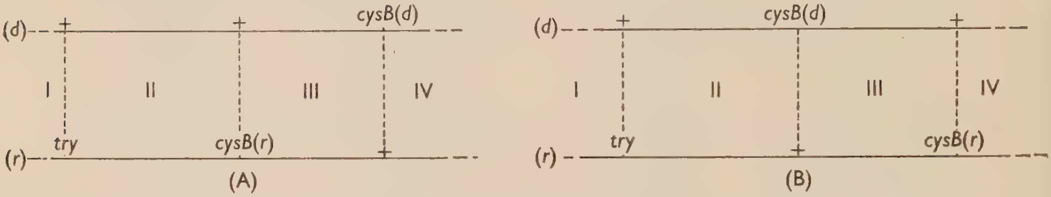

Fig. 1. Schematic representation of synapsis during transduction of a *try.cysB* double mutant (recipient) with phage propagated on another *cysB* mutant (donor). Part of the chromosome of the bacterial recipient is shown in (*r*) and the homologous part of the chromosome of the donor strain, introduced by the phage, is shown in (*d*); the mutational sites are denoted as *try*, *cysB*(*r*) and *cysB*(*d*) and their corresponding wild-type sites as +. (A) and (B) represent alternative arrangements of the *cysB* markers of donor and recipient.

When *try.cysB* double mutants are treated with phage grown on wild-type cells and plated on minimal medium plus tryptophan, the ratio of the numbers of prototrophic to tryptophanless colonies is between 0·4 and 0·7 (Demerec & Demerec, 1956; Table 6). This ratio indicates the relative frequency of simultaneous incorporation of the wild-type alleles of the *try* and *cysB* markers, and of incorporation of that of the *cysB* marker only. This ratio would also be expected in crosses of the type *try.cysB*(*r*) (recipient) with *cysB*(*d*) (donor), when the order of markers is *try-cysB*(*r*)–*cysB*(*d*) (Fig. 1 A), although the actual numbers of both classes would be expected to be proportionally reduced, since *cysB*(*r*) and *cysB*(*d*), between which crossing-over is required, will be closely linked (Table 1). However, if the *cysB*(*d*) mutational site is located between the *try* and *cysB*(*r*) sites, (Fig. 1 B) prototrophs can arise only as a result of crossing-over in each of the regions I–II–III–IV (Demerec & Demerec, 1956); whereas, crossing-over in two only of these four regions (III–IV) would give rise to tryptophanless (*try*.*cysB*$(d)^+$–*cysB*$(r)^+$) colonies. Since quadruple crossing-over is believed to be less frequent than double crossing-over, when the order of the markers is as shown in Fig. 1 B, the ratio of prototrophic to tryptophanless cells should be much less than the corresponding ratio when the order is as shown in Fig. 1 A, or when the phage has been propagated on the wild-type strain. The three-point crosses shown in Table 6 thus allow the order of the various sites of mutation within the *cysB* locus to be inferred with a much greater degree of precision than is possible from the results of two-point crosses of the type shown in Table 1.

DISCUSSION

Pseudoallelism and arrangement of loci. The data shown in Tables 1–4 support the conclusions of Demerec *et al.* (1954) that in Salmonella there are functionally undifferentiated regions in the genetic material, which are, however, divisible by mutation and recombination, as has been observed in certain other organisms (Pontecorvo, 1952; Benzer, 1955). Tests by abortive transduction gave no evidence of complementarity and thus of functional subdivision, within any group of mutants, and make it seem likely that with the exception of *cys-48* and *-50*, the cysteineless mutants of each group established by nutritional tests which have been found to correspond to a similar group by transductional tests, have their mutational sites within the same gene locus. The exclusion of linkage greater than *c.* 2% between *cysC-7* and *cys-48* (or *cys-50*) by donor-phenotype selection (Table 5), suggests that the genetic markers of *cys-48* and *-50* are unlinked to those of the other mutants of similar phenotype (*cys-*D_1). *Cys-48* and *-50* may thus arise by mutation in a completely separate locus, which is, however, not supported by their behaviour in nutritional and syntrophism tests already noted, nor by further tests which were unsuccessful in demonstrating syntrophy between these two mutants and others of the *cys*D_1 phenotype. Alternatively, their genetic markers may be linked to those of the other *cysD* mutants, and in the same locus in the intact bacterial genome, but that this locus is invariably fragmented at a point between the markers of *cys-48*, *-50* and the other *cysD* mutational sites during the incorporation of bacterial genetic material into phage particles.

The genetic markers of the cysteineless mutants used in this study appear therefore to be located in five, (or possibly six), gene loci controlling cysteine biosynthesis from sulphate; one locus controlling the reduction of sulphate to sulphite, and not more than two other loci controlling the reduction of sulphite to thiosulphate (Clowes, 1958). This is in contrast to the conclusions of Hockenhull (1949) using *Aspergillus nidulans*, where heterokaryon studies suggested that this synthesis was controlled by six loci, three of which control the reduction of sulphate to sulphite, and three others of which control the reduction of sulphite to thiosulphate. However, in the *cysC* and *cysD* groups, the detection of abortive transduction is rendered technically difficult by the high background growth of the mutants of these groups on minimal agar, so that further subdivision of these loci is not conclusively eliminated.

Only one of these five loci (*cysB*) is linked to the array of loci which have been found to control tryptophan biosynthesis (Demerec & Demerec, 1956). The data shown in Tables 4 and 5 indicate that, among the remaining four loci, two only show linkage to each other (*cysC* and *cysD*) and no linkage, has been demonstrated to any other locus. There is no parallel, therefore, in this system to that demonstrated in tryptophanless mutants (Demerec & Hartman, 1956) and histidineless mutants (Hartman, 1956), where there was close linkage of all the loci concerned, and where the genetic order of these loci was found to follow the same sequence as the synthetic reactions in the biochemical pathway which are under their control. However, it is of interest to note that the two linked loci, *cysC* and *cysD*, would appear to be concerned in consecutive reactions in the cysteine biosynthetic pathway (Clowes, 1958).

Extent of mutational changes. There have been several reports of pairs of mutants which behave as members of the same transduction group but which do not recombine to yield prototrophs by transduction (Demerec, 1955; Hartman, 1956; Yura, 1956; Demerec & Hartman, 1956). It has been suggested that these pairs of mutants either are duplicates, i.e. reoccurrences of mutation at the same site, or that their mutational sites are so near that crossing-over does not occur (Demerec, 1956*b*), or alternatively occurs too infrequently to be detected.

In other instances a mutant, which by its nutritional character and behaviour in certain transductions appears to fall in with a certain group of mutants, fails to form prototrophs by transductions with any one of a number or all of the members of this group.

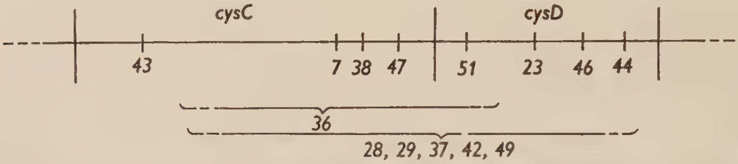

Fig. 2. Probable arrangement of mutational sites in the *cysC* and *cysD* loci. Vertical lines represent approximate location of 'single-site' mutants. 'Multiple-site' mutants are represented by a bracket covering the extent of the apparent mutation. The exact order within the two groups of mutants *cysC-7*, *-38* and *-47*, and *cysD-23*, *-46* and *-44* is not known.

This phenomenon has been interpreted on the theory that failure to recombine to produce prototrophs by transduction, indicates identity (or overlap) of the mutational sites involved. This requires that in these mutants, at each site corresponding to that at which mutation has occurred in all other mutants which fail to produce prototrophs, there is also a mutational change. Since simultaneous but independent mutations at several sites is considered to be so infrequent as to be excluded, it is suggested that there is a simultaneous dependent mutation which extends so as to overlap all these sites. Such an event could result from a chromosomal aberration such as a deletion or an inversion. This type of mutant is, in general, less frequently isolated than the zero pairs, the only examples so far reported in *Salmonella* being *cysA-20* (Demerec, 1955); *hiB-22* (Hartman, 1956), and *athA-10* (Yura, 1956). Similar mutants have been reported in bacteriophage T2 by Benzer (1955).

The situation existing in the linked loci *cysC* and *cysD* (Table 2) suggests that many such mutations occur in these loci. It is assumed that the data in Table 2 reflect the mapping of mutational sites shown in Fig. 2, i.e. that *cysC-43*, *-47*, *-38* and *-7*, and *cysD-51*, *-23*, *-44* and *-46* are 'single-site mutants' whose markers are situated within the two linked loci *cysC* and *cysD*, but that *cysD-28*,

-29, *-37*, *-42*, and *-49* behave genetically as though in them the mutational site extended along the whole of the *cysD* locus and part of the *cysC* locus, for they are unable to produce prototrophs by transduction, both among themselves and with all the 'single-site' mutants except *cysC-43*. Similarly, *cysD-36* behaves as though the mutation extended over part of the *cysD* locus including the site of *cys-51*, and a part of the *cysC* locus including all the mapped sites except that of *cys-43*. From the data, the relative order of the mutational sites of single-site mutants cannot be fixed except that since *cysC-43* produces prototrophs with the multiple site mutants, its marker is likely to be at the end of the *cysC* locus remote from the *cysD* locus and since *cysD-23*, *-46* and *-44* produce prototrophs with *cysD-36*, their markers are likely to be more distant from the *cysC* locus than is *cysD-51*. However, the order of markers within the two groups (*cysC-7*, *-38*, and *-47*) and (*cysD-23*, *-44*, and *-46*) is not known. These genetic data are supported biochemically by the correspondence of the 'single-site' mutants to those of the biochemical group D_1, which will stimulate by syntrophy all *cysA* and *C* mutants, and the 'multiple site' mutants to those of biochemical group D_2, which are unable to feed any of these same strains (Clowes, 1958). It has been suggested that the *cysC* and *cysD* loci control reactions which are sequential in the biosynthesis of cysteine, and that the step controlled by the *cysD* locus follows that controlled by the *cysC* locus. Both classes of mutants can grow on thiosulphate, but *cysC* mutants are able alternatively to utilize sulphite (Clowes, 1958). If $cysD_2$ mutants are deficient of the functions controlled by both *cysC* and *cysD* they would be expected to have the same nutritional requirements as $cysD_1$ mutants, but they would not accumulate the presumptive sulphite-like intermediate necessary to feed *cysC*. The difference in phenotype of D_1 and D_2 mutants previously noted (Clowes, 1958) can thus be explained by the genetic relationships shown in Fig. 2 where the D_1 mutants are represented as 'single-site' mutants located within the *cysD* locus, and the D_2 auxotrophs are shown as 'multiple-site' mutants whose site of mutation extends over parts of both the *cysD* locus and the closely linked *cysC* locus, resulting in a simultaneous loss of the two functions controlled by the two loci.

In this region therefore in six of fourteen mutants in the two linked groups *cysC* and *cysD*, the mutational site appears to cover a region extending over more than one gene locus. None of these six strains showed any reversion to prototrophy. If the reversions observed in the case of the single-site mutants of this region are true 'back mutations' which restore the original unmutated condition, this suggests that the mutation in these 'multiple site' strains may be a true chromosomal deficiency. Within the limits of our data, it seems that the mutation may extend over the same region in all mutants except *cysD-36*, covering all known sites in the *cysD* locus and terminating at one end between the cluster of markers *cysC-7*, *-38*, and *-47* and the marker of *cysC-43*, and at the other end at a point probably within the *cysD* locus.

Yanofsky (1957) has found evidence for similar phenomena among a series of mutants of *Escherichia coli* selected as resistant to T1 phage and found to have a concomitant requirement for tryptophan. The genetic analysis of these mutants is supported by more extensive biochemical data than has been possible in the present studies. The loci controlling the four or five consecutive steps of synthesis of tryptophan from anthranilic acid appear in this organism to be closely linked and to be arranged in the same sequence as the biochemical reactions under their control, as was found in Salmonella (Demerec & Hartman, 1956). Genetic tests indicate that the T1-resistant tryptophan-requiring auxotrophs may result from deletions covering two or more of these loci, and enzyme isolations revealed that the corresponding enzymes controlled by these loci are in fact absent from the cell-free extracts of these mutants.

The high frequency of isolation and the correspondence of the mutational region in the multiple-site *cysC.cysD* mutants is so far unique amongst Salmonella auxotrophs. It may be that the genetic material is unusually fragile at these points so that 'multiple-site' mutations arise more frequently in this region than elsewhere. However in Drosophila it has been suggested that deletions may be a common form of genetic change (Demerec, 1956*b*). If this situation has a parallel in bacteria, it is possible that the type of deletion seen in the *cysC–cysD* region occurs frequently in all parts of the genetic material, but that in most instances this type of change has a lethal effect, an exception being in the *cysC–cysD* region. The auxotrophs used in this and in related studies in Salmonella are those in which mutations have occurred in loci controlling the biochemical synthesis of an amino-acid, vitamin or purine. These mutants were isolated in the first instance by their inability to grow on a minimal medium which supports the growth of the wild-type strain whilst growing on a complete nutrient medium. They were then further selected by their requirement for a single amino-acid, vitamin or purine (or for certain specific combinations of these growth factors) to be added to the minimal medium. Any deletion of genetic material resulting in the loss of several adjacent but functionally unrelated loci, would be expected in most instances to have a lethal effect under these conditions of selection. However, in any locus (or in any series of functionally related and adjacent loci) of such an extent that deletions of a comparable size could occur within the locus (or loci), their effect would not be lethal in the presence of the specific growth factor or of a suitable precursor. Thus it might be expected that the frequency with which deletions have a lethal effect bears an inverse relationship to the physical size of the locus, (or adjacent related loci).

An indication of the relative extent of various loci in Salmonella can be found from a comparison of the maximum crossing-over occurring between any two mutants of each locus. An estimate of these values can be obtained from the mean of the figures for reciprocal crosses of mutants as shown in Tables 1–3. These maximum values are for *cysB*, 30 %; *cysC*, 69 %; *cysD*, 37 % (excluding *cys-48* and *-50*); and for *cysE*, 28·5 %. The comparable figure for *cysA* (Demerec, 1955) is calculated as 28 %. A similar value obtained for the extent of the two linked loci *cysC–cysD* is 97·5 %, whilst the maximum figure within the four linked tryptophan loci is 37 % (Demerec & Demerec, 1956) and that within the four linked histidine loci is 76·9 % (Hartman, 1956). These values suggest that the loci *cysC* and *cysD* may extend over a longer segment of the chromosome than that covered by other loci or by other groups of adjacent, related loci and may well account for the frequency with which multiple-site mutants are found in the *cysC-cysD* region. This would also suggest that the deletions shown in Fig. 2 are limited to within the *cysC* and *cysD* loci.

Fine structure of the cysB locus and its relation to phenotype. In Table 6, from crosses involving *tryB-2.cysB-45* as recipient, the ratios of prototrophic to tryptophanless cells are similar when the phages are derived from either *cysB-10* or *cysB-15*, to the value found when the phage is derived from wild-type cells (0·4–0·7). This suggests the order *tryB-2–cysB-45–cysB*(*-10*, *-15*), the use of ( ) brackets in all cases implying no particular order of those markers within the brackets. In contrast, when the phages result from lysis of either *cysB-18* or *cysB-24* this ratio is reduced (0·09, 0·12), suggesting the order *tryB-2–cysB*(*-18*, *-24*)*–cysB-45–cysB*(*-10*, *-15*). Similarly, from the crosses with *tryD-11.cysB-18* as recipient (Table 6) the sequence *tryD-11–cysB-14–cysB-18–cysB*(*-10*, *-15*) is most indicated. In combination the order (*tryD-11–tryB-2*)–*cysB-14–cysB-18–cysB-45–cysB*(*-10*, *-15*) is given. Other experiments using either *tryD-11.cysB-12*, *tryA-8.cysB-40* or *tryA-8.cysB-41* as recipients and crossed with *cysB-14*, *-18*, *-10* and *-15* as donors give results analogous to the crosses shown in Table 6 using *tryB-2.cysB-45* as recipient and suggest the order

(*tryA-8*, *tryB-2*, *tryD-11*)–*cysB-14–cysB-18*
–*cysB*(*-12*, *-40*, *-41*, *-45*)–*cysB*(*-10*, *-15*).

This sequence of markers within the *cysB* group separates the locus into at least four regions with respect to the biochemical heterogeneity, since, as already reported, *cysB-12*, *-14*, *-15*, *-40*, *-41* and *-45* are alike in being unable to replace the cysteine requirement with equivalent amounts of sulphur either as sulphite or as thiosulphate (phenotype B_1), whereas the phenotype of *cysB-18* permits slow adaptation to sulphite utilization (phenotype B_2) and that of *cysB-10* permits growth on either sulphite or thiosulphate (phenotype B_3).

Crosses using *cysB-24* or *cysB-25* as donor with each of the double mutants having one of the markers *cysB-12*, *-40*, *-41*, or *-45* as recipient, and similar to the cross shown on line 3, Table 6, indicate the order

try–cysB(*-24*, *-25*)*–cysB*(*-12*, *-40*, *-41*, *-45*).

Unfortunately double mutants of either *cysB-24* or *-25* were not available, and it has been assumed that these markers form a cluster with *cysB-18* and are not separated by *cysB-14*. Further crosses of the type shown in lines 6 and 7, Table 6, using *tryD-11.cysB-18* as recipient and either *cysB-4*, *-16*, or *-27* as donor indicate the order *try–cysB-18–cysB*(*-4*, *-16*, *-27*). It has been assumed that these markers (*cysB-4*, *-16*, *-27*) form a similar cluster with the other markers of the B_1 phenotype (*cysB-12*, *-40*, *-41* and *-45*) and are not separated by *cysB-10*.

No evidence from three-point crosses is available to map the relative order of *cysB-10* and *-15* in the same manner. The crosses shown in Table 6 using these two mutants as donors, suggest that the markers involved in each of the four crosses can be represented diagrammatically as in Fig. 1A, where *cysB*(*r*) represents either *cysB-45* or *cysB-18*, and *cysB*(*d*) represents either *cysB-15* or *cysB-10*. The relative distances between the *cysB* markers of donor and recipient may then be measured by the crossing-over within region III. In these crosses, three phenotypes can be identified in the presence of the recipient phenotype, namely, wild-type (crossing-over I–III), *try* (crossing-over II–III) and *cysB* (crossing-over I–II; I–IV or I–II–III–IV). Since quadruple crossing-overs (I–II–III–IV) are considered to be less frequent than double crossing-overs, a close approximation of the frequency of crossing-over in region III is given by the ratio of the numbers of wild-type colonies + tryptophanless colonies to the total number of wild-type + tryptophanless + cysteineless colonies. These ratios are shown in the last column. Table 6. In both crosses involving *cysB-15*, this ratio is found to be greater than in the two crosses involving *cysB-10*, and suggests that the former marker is at the greater distance from either *cysB-18* or *cysB-45*. It is suggested, therefore, that the order of markers shown in Fig. 3 represents the most probable disposition

Table 6. *Numbers of prototrophic and singly auxotrophic colonies obtained in transductions of a try.cysB double auxotroph with phage raised on other cysB mutants*

		Numbers of colonies of phenotype				
Recipient bacteria	Source of phage	$try^{+}.cys^{+}$ (prototrophic)	$try.cys^{+}$ (tryptophanless)	$try^{+}.cys$ (cysteineless)	Ratio of prototrophs: $try.cys^{+}$	Ratio of prototrophs + $try.cys^{+}$: total
1. *tryB-2.cysB-45*	*cysB-10*	4	14	400	0·501	0·025
		13	17	1212		
		9	17	1190		
		11	23	1532		
2. *tryB-2.cysB-45*	*cysB-18*	2	27	910	0·09	—
		7	75	1780		
		6	64	—		
		7	74	1877		
3. *tryB-2.cysB-45*	*cysB-24*	1	10	490	0·12	—
		4	36	1115		
		2	16	—		
		4	33	1065		
4. *tryB-2.cysB-45*	*cysB-15*	7	13	382	0·61	0·062
		44	100	3100		
		56	66	2740		
5. *tryD-11.cysB-18*	*cysB-14*	1	20	1202	0·08	—
		2	11	1295		
		1	17	1290		
6. *tryD-11.cysB-18*	*cysB-15*	30	60	205	0·67	0·083
		35	70	1520		
		43	40	1346		
7. *tryD-11.cysB-18*	*cysB-10*	84	172	7300	0·49	0·034
8. *tryB-2.cys-45*	Wild-type	311	599	557	0·58	—
		312	344	419		
9. *tryD-11.cysB-18*	Wild-type	227	561	—	0·65	—
		236	611	—		

Numbers of colonies are those which appeared on a total of five plates after plating 2×10^8 bacteria with 5×10^9 phage (multiplicity 25) on each plate, and which were classified phenotypically by replica plating.

of the various sites within the *cysB* locus, and separates this locus into at least four and probably not more than five regions of secondary heterogeneity.

The inability of the biochemical tests to differentiate between the phenotypes of any of the $cysB_1$ mutants and the close linkage of all mutants in the *cysB* group make it appear unlikely that there are five distinct but closely linked loci. This was confirmed by the failure to demonstrate abortive transductions between mutants representative of the various *cysB* phenotypes, although 'minute' colonies resulting from abortive transduction could be clearly seen when any of these mutants were treated with lysates of either wild-type donors or other auxotrophs, including those of the *cysA*, *cysC*, *cysD* and *cysE* groups. The phenomenon of abortive transduction has so far been explicable by a '*cis:trans*' effect, the wild-type phenotype being dependent upon the presence of a supernumary fragment of genetic material, which does not carry a mutation within the same functional locus as that in the chromosome of the recipient cell. Since the phenotypic differences found within the *cysB* locus are difficult to explain by leakiness or other quantitative variations, it appears that qualitative differences may exist between the mutants of the same gene locus. Other such examples of secondary biochemical heterogeneity among mutants within the same gene locus have been cited by Demerec (1956*b*).

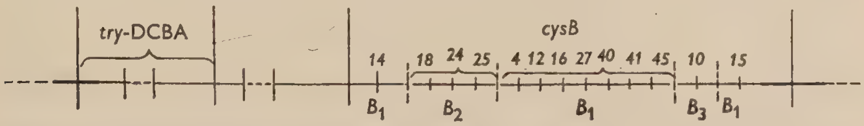

Fig. 3. Probable arrangement of mutational sites in the *cysB* locus. Vertical lines represent sites of mutation and when several are included within one bracket the relative order within the bracket is doubtful. B_1, B_2 and B_3 represent different phenotypes within the *cysB* locus.

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Studies on the Cell-Wall Composition and Taxonomy of Actinomycetales and Related Groups

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SUMMARY: The cell-wall compositions of 51 strains of *Actinomyces*, *Nocardia*, *Streptomyces*, *Micromonospora*, *Mycobacterium* and *Propionibacterium* have been investigated, together with those of 7 strains of Eumycetes. The cell walls of the actinomycetes were made up of sugars, amino sugars and amino acids (the latter few in number). The general pattern of components was thus identical with that previously found for Gram-positive bacteria. In the fungi, however, the mycelial walls were composed entirely of carbohydrate. These results suggest that the actinomycetes are not related to the fungi but should be classified with the bacteria proper. On the basis of cell-wall composition a classification of the actinomycetes is proposed, to consist of 3 families: Mycobacteriaceae containing the genera *Mycobacterium*, *Nocardia* and *Corynebacterium*; Streptomycetaceae containing *Streptomyces* and *Micromonospora*; and Actinomycetaceae with a single genus *Actinomyces*. The propionibacteria are probably related to *Streptomyces*. It is also suggested that a separate order Actinomycetales is unnecessary and that the families proposed can more suitably be included in the Eubacteriales.

Mycelium formation has always been the primary criterion for the inclusion of any species in the actinomycetes, but it is not always an easy one to apply as can be seen from the large number of different classifications proposed for these organisms in the last 50 years; Waksman (1950) outlined 14 in his monograph, and this is not an exhaustive list. There has been much disagreement, for example, about the position of corynebacteria and mycobacteria. Jensen (1931) thought that both should be included in the actinomycetes, while Stanier & van Niel (1941) proposed that they should be omitted; Waksman & Henrici (1943), adopting a middle course, excluded *Corynebacterium* from the Actinomycetales, but included *Mycobacterium*.

Apart from the uncertainty about which genera shall be included in the actinomycetes, there is considerable divergence of view about the arrangement and naming of those which are by common consent admitted. This point is well brought out by comparing Waksman & Henrici's (1943) scheme, involving the four genera *Actinomyces*, *Nocardia*, *Streptomyces* and *Micromonospora*, with that adopted by *Topley & Wilson's Principles*, 4th edition (1955) in which there is only one, *Actinomyces*. Although the latter classification is specifically stated to be a provisional one, the disparity between the two systems shows how much uncertainty still exists in the classification of these organisms.

The present paper records the results of an investigation into the cell-wall compositions of actinomycetes of different types, and discusses the application of the findings to the classification of these organisms. Our previous work (Cummins & Harris, 1956*a*, *b*) has indicated that the composition of the bacterial cell wall is a character of some importance in classification and a preliminary investigation on a small number of strains of actinomycetes (Cummins & Harris, 1956*c*) suggested that the same held good in this group also. Two recent papers, by Romano & Sohler (1956) on the differentiation of *Streptomyces* and *Nocardia* by cell-wall composition, and by Hoare & Work (1957) on the distribution of stereoisomers of diaminopimelic acid in Actinomycetales, have also indicated the value of this type of analysis in the taxonomy of micro-organisms. We have confined our attention entirely to Gram-positive species, and have not examined Gram-negative filamentous organisms such as *Streptobacillus moniliformis* or *Fusiformis necrophorus* (*B. funduliformis*).

METHODS

Naming of strains

We have in general used the names proposed by Waksman & Henrici (1943), and therefore references in the present paper to *Actinomyces*, *Nocardia*, *Streptomyces* and *Micromonospora* have the meanings which those authors gave to them. However, in the case of any particular strain we have used the name the strain bore when we received it, whether or not this is in agreement with Waksman & Henrici's system. This has meant, for example, that about half of the microaerophilic strains are referred to as *Actinomyces israelii* and not as *A. bovis* and that in the case of some of the aerobic saprophytes the genus is given as *Actinomyces* and not *Streptomyces*. We have not investigated the cultures in detail ourselves except to check them for purity.

Sources of strains

Actinomyces bovis *and* A. israelii. Details of the sources of these strains are given in Table 1.

Streptomyces *and* Actinomyces (*aerobic*). All from National Collection of Type Cultures (NCTC) or The National Collection of Industrial Bacteria (NCIB).

Micromonospora. *Micromonospora* sp. LSTM from London School of Hygiene and Tropical Medicine; *Micromonospora* sp. G1, from Dr M. R. J. Salton, Department of Bacteriology, University of Manchester; *M. chalceae*, strains S3, S5 and C from Dr H. L. Jensen, Statens Planteavls Laboratorium, Lyngby.

Nocardia. *N. caprae*. Stock strain of London Hospital Bacteriology Department, origin unknown; *N. leishmanii* 658, originally obtained as NCTC 658 and now discarded from National Collection of Type Cultures, as the original description was inadequate; *N. gardneri* NCTC 6531, *N. asteroides* NCTC 6761 and *N. farcinica* NCTC 4524 from National Collection of Type Cultures.

Mycobacterium. *M. tuberculosis* B.C.G. This strain was obtained from Dr E. Lederer, Institut Pasteur, Paris, as the bacterial residue after extraction of the organisms with alcohol + ether and chloroform. The material showed apparently intact bacilli which still stained acid-fast. *M. tuberculosis* 'C' (human strain) obtained as formalin killed culture from Dr A. B. Paterson, Central Veterinary Laboratory, Weybridge. *M. phlei* NCTC 8156 and *M. smegmatis* NCTC 8159 from National Collection of Type Cultures.

Propionibacterium. All five strains were obtained from the National Collection of Industrial Bacteria.

Table 1. *Sources of strains of* Actinomyces bovis *and* A. israelii

Name of organism	No. or name of strain	Source
Actinomyces bovis	NCTC 9429	Isolated from cattle in U.K. by Mr M. B. Hawksley, Bayer Biological Institute
	NCTC 9430	
	NCTC 9431	
	NCTC 4500	Isolated from cattle in Australia by Professor Carne (see Erikson, 1940)
	NCTC 4501	
	NCTC 4502	
	2791	Isolated from cattle in U.K.; received from Mr Gordon Frazer, Royal (Dick) School of Veterinary Studies, Edinburgh
	975	
	B.10	From Professor R. Lovell, Royal Veterinary College. Bovine strains isolated in U.K.
	622	
	ATCC 10048	Isolated from pleural fluid in human case
	ATCC 12597	Origin unknown
A. israelii	NCTC 9765	Isolated in London Hospital from clinical case of actinomycosis
	ATCC 12102	Isolated from brain abscess in human case
	ATCC 12103	Isolated from human case of cervico-facial actinomycosis
	Bowdery	8 strains received from Professor L. P. Garrod, St Bartholomew's Hospital. All isolated from human cases of actinomycosis
	McGrath	
	Duncton	
	Hall	
	Kattenhorn	
	Goldworthy	
	Ingram	
	Denning	

Fungi. The strains of *Mucor mucedo* 26441 and *Rhizopus stolonifer* 42844 were obtained from the Commonwealth Mycological Institute, Kew. Other fungi were stock strains of the Bacteriology Department, London Hospital.

Culture media

Solid media were not used because of the tendency of actinomycetes to produce a very adherent growth.

Actinomyces israelii *and* A. bovis. These species were grown in screw-capped bottles of glucose serum broth at 37°. The medium was prepared by adding 5 ml. of sterile 20% (w/v) glucose and 5 ml. sterile horse serum to 100 ml. of tryptic digest broth.

Strains of *Streptomyces* and *Nocardia*, and aerobic strains of *Actinomyces*, were grown at 30° in 1% (w/v) glucose digest broth; 200 ml. amounts of medium were used in 1500 ml. conical flasks to give good aeration.

Micromonospora. The same medium was used as for *Streptomyces*, but because of the slow growth of these strains the normal procedure was modified.

A preliminary culture of 10 ml. of glucose digest broth in a 50 ml. conical flask was incubated at 30° for 3–4 days. The granular growth was then lightly ground in a TenBroeck tube under sterile conditions, and pipetted back into the original flask. The dispersed fragments grew rapidly and after another 3 or 4 days the whole 10 ml. of culture was used to inoculate 200 ml. of medium in a 1500 ml. flask, which was then incubated for a further 10 days.

Mycobacterium. *M. phlei* and *M. smegmatis* were grown in 5% (v/v) glycerol broth at 37°. *M. tuberculosis* H37Rv was grown in Tween-Dubos liquid medium.

Propionibacterium spp. These strains were grown in tomato-juice broth (Briggs, 1953) at 37° in an atmosphere of hydrogen + 10% (v/v) CO_2.

Fungi. The strains of fungi were grown in glucose (1%, w/v) digest broth in conical flasks at 30°. The strains of *Penicillium*, *Aspergillus*, *Mucor* and *Rhizopus* grew rapidly and the cultures were killed by formalin after 24–48 hr. The pathogenic species grew more slowly and 10–14 days of incubation were necessary to obtain sufficient growth.

Preparation of cell-wall fractions

All cultures were killed by the addition of formalin (40%, w/v, HCHO) to a final concentration of 0·5%. This was allowed to act at room temperature for 24 hr. and the organisms then removed by centrifugation and washed twice in distilled water. The cell-wall fractions were prepared as previously described (Cummins & Harris, 1956*a*), except that in many cases it was found an advantage to break up firm nodules of growth by grinding the suspension thoroughly in a TenBroeck tube before disintegration. This preliminary grinding was essential in the case of fungal hyphae which otherwise were too long and coiled to be disrupted by the ballotini beads.

It is probable that in preparations from strains of *Streptomyces* and *Micromonospora*, particularly the latter, spore walls as well as mycelial walls were present, and the same is true of fractions from *Mucor* and *Rhizopus* where zygospore formation had occurred before the cultures were killed. The results obtained, however, suggest that either the number of spore-wall fragments present in each case was very small or that they have the same qualitative composition as the mycelial walls. The latter was certainly the case in a purified spore preparation from a strain of *Streptomyces* (see below).

Chromatography

The methods of hydrolysis and chromatography of cell-wall fractions were those described previously (Cummins & Harris, 1956*a*). Separation of the stereoisomers of diaminopimelic acid (DAP) was as described by Hoare & Work (1957).

RESULTS

The method of recording results is the same as that used in previous papers (Cummins & Harris, 1956*a*, *b*). The relative amounts of the different substances present in chromatograms have been arbitrarily scored as + + +, + +, +, ±

or trace; as before we have recorded only major components in the case of the amino acids. The substance we had previously called 'unknown hexosamine' has now been isolated and characterized by Strange (Strange, 1956; Strange & Dark, 1956; Kent, 1957). We have referred to it in Tables 2–5 by the name 'muramic acid' which Strange has suggested for it (see Work, 1957). The cyclic peptide of lysine and aspartic acid (Swallow & Abraham, 1957; Abraham & Swallow, personal communication), which we had previously found in lactobacilli occurred also in hydrolysates from some strains of *Actinomyces bovis* and is called aminosuccinoyllysine in the Tables. It almost certainly does not occur as such in cell walls but is formed by cyclization during acid hydrolysis. Its presence, however, probably indicates that the lysine and aspartic acid residues are linked in a specific way in the original material. It is not found, for example, in hydrolysates of those strains of *Lactobacillus bifidus* whose cell walls contain both aspartic acid and lysine (Cummins, Glendenning & Harris, 1957).

The chromatographic method used separates LL-diaminopimelic acid from the DL- and DD- isomers, but the two latter run to the same position. They can be further differentiated by enzymic methods but this has not been done in the present instance. In fact DD-diaminopimelic acid seems to be very uncommon, and it has so far only been reported in the whole cells of strains of *Micromonospora* by Hoare & Work (1957). Hoare & Work also described an unknown substance which gave the same greenish colour with ninhydrin as diaminopimelic acid in the special solvent used to separate the isomers, but which ran more slowly than any of them. We have found a substance with similar properties in the cell walls of two strains of *Micromonospora*; it is recorded in Table 3 as 'slow-moving component'.

In certain strains of *Actinomyces bovis* an unidentified sugar was found. This moved faster than rhamnose in phenol/water, lutidine/water and butanol/acetic acid/water and gave a colour reaction with aniline hydrogen phthalate very similar to the methylpentoses rhamnose and fucose. It is referred to in Table 2 as sugar component P.

Actinomyces israelii *and* A. bovis

The first point that emerges from a study of the results in Table 2 is the uniformity of cell-wall composition in the strains of *Actinomyces israelii*. All the 11 strains so named, together with two of those called *A. bovis* (ATCC 10048, ATCC 12597) seem to form a well-defined natural group in which the principal cell-wall components are galactose, glucosamine, muramic acid, alanine, glutamic acid and lysine. It was noted during the preparation of suspensions for disintegration that the growth of members of this group in glucose-serum broth was very uniform in type, and consisted of firm rounded or irregular masses at the bottom of an otherwise clear medium. After incubation had been continued for several days there was a tendency for looser floccules to develop but this did not destroy the characteristic 'breadcrumb' appearance which has been described previously as typical of microaerophilic parasitic actinomyces from human sources (Colebrook, 1920; Erikson, 1940; Rosebury, 1944; and others).

Table 2. *Cell-wall composition in* Actinomyces israelii *and* A. bovis

Name of strain	Arabinose	Rhamnose	Galactose	Glucose	Mannose	Fucose	Sugar component 'P'	Glucosamine	Galactosamine	Muramic acid	Aspartic acid	Alanine	Glutamic acid	Lysine	Glycine	Isomers of diamino-pimelic acid		Slow-moving component	Aminosuccinoyllysine
																LL	DL		
Actinomyces israelii, 8 strains from Professor Garrod (see Table 1)	–	–	++	–	–	–	–	+	–	+	·	+++	+++	+++	·	·	·	·	·
A. israelii 3 strains: NCTC 9756; ATCC 12102; ATCC 12103	–	–	++	–	–	–	–	+	–	+	·	+++	+++	+++	·	·	·	·	·
A. bovis ATCC 10048	–	–	++	–	–	–	–	+	–	+	·	+++	+++	+++	·	·	·	·	·
A. bovis ATCC 12597	–	–	++	–	–	–	–	+	–	+	·	+++	+++	+++	·	·	·	·	·
A. bovis NCTC 9429	–	++	+	–	Tr.	–	++	+	–	+	+++	+++	+++	+++	·	·	·	·	++
A. bovis NCTC 9430	–	++	Tr.	–	Tr.	++	++	+	–	+	+++	+++	+++	+++	·	·	·	·	++
A. bovis NCTC 9431	–	++	+	–	–	–	++	+	–	+	+++	+++	+++	+++	·	·	·	·	++
A. bovis 2791	–	++	–	–	–	++	++	+	–	+	+++	+++	+++	+++	·	·	·	·	++
A. bovis 975	–	+	–	–	–	+	+	+	–	+	+++	+++	+++	+++	·	·	·	·	++
A. bovis B. 10	–	?Tr.	–	–	–	?Tr.	–	+	–	+	+++	+++	+++	+++	·	·	·	·	++
A. bovis NCTC 4501	++	–	+	–	Tr.	–	–	+	–	+	·	+++	+++	·	·	·	(+++)*	·	·
A. bovis 622	+	–	±	–	–	–	–	+	–	+	·	+++	+++	·	·	·	+++	·	·

* D.A.P. present: assumed to be DL-isomer.

Tr. = trace.

The results of cell-wall analysis of the strains of *Actinomyces bovis*, on the other hand (i.e. except ATCC 10048 and ATCC 12597), do not show the same homogeneity, and at least 2 subgroups can be recognized. The larger subgroup comprises 6 strains (B10, 975, 2791; NCTC 9429–31) all isolated from cattle in various parts of the United Kingdom. The pattern of amino acids in the cell wall of these 6 strains (aspartic acid, lysine, glutamic acid and alanine) is identical with that previously found in various strains of lactobacilli (Cummins & Harris, 1956*a*), and hydrolysates also contained the acid-resistant cyclic peptide mentioned above. The cell walls of these strains of *A. bovis* were also characterized by the presence of rhamnose and of the unidentified sugar component (P). In addition, 3 of the 6 strains contained fucose. This unusual combination of amino acids and sugars made them easily identifiable. The growth of these strains in broth gave a generalized turbidity and a fine granular deposit, quite unlike that of the strains of *A. israelii*. None of them grew freely, and in the case of strain B10 there was insufficient material for a full analysis of the sugar components. There was no doubt, however, that B10 belonged to this subgroup.

Other organisms received as *Actinomyces bovis* were 3 strains examined by Erikson (1940) and originally isolated in Australia. Two of these (NCTC 4500 and NCTC 4502) had identical cell-wall compositions with an amino acid pattern unusually complex for Gram-positive bacteria since eight amino acids were present in relatively large amounts. These were aspartic acid, glutamic acid, serine, glycine, alanine, lysine, leucine and phenylalanine. The spots for alanine, glutamic acid and lysine were rather more intense than those for other amino acids. Glucosamine and muramic acid were present in cell-wall fractions from both strains, with rather smaller amounts of galactosamine. The results for these two organisms have not been incorporated in Table 2 to avoid complicating it unduly. The third strain, *A. bovis* NCTC 4501, is apparently a corynebacterium since cell-wall fractions from it contain arabinose, galactose, alanine, glutamic acid and diaminopimelic acid. Similar results were obtained with cell-wall fractions from strain 622, and it is very probable that this also is a diphtheroid. It was somewhat surprising to find that strain 4501 was very different from 4500 and 4502, since Erikson found that suspensions of all 3 were agglutinated to similar titres by a serum prepared against one of them (Erikson, 1940; see p. 38 of her monograph).

The present results fully support the conclusions of Erikson (1940) and Thompson (1950) among others, that human and bovine strains of *Actinomyces* are distinct. To avoid the anomalous position created by having to call a predominantly human parasite by the specific term *bovis*, Erikson suggested that it would be reasonable to use the name *A. israelii* (originally proposed by Kruse, 1896), for strains of human origin which correspond in description to the organisms originally isolated by Wolff & Israel (1891), and to regard strains of bovine origin as a distinct species, *A. bovis*. If our results are at all representative, however, there is very little justification for placing bovine strains in the same genus as strains of *A. israelii*. In fact, of the 12 strains received as *A. bovis*, 2 (10048 and 12597) are identical with the human strains, 2 (4500 and

4502) show a cell-wall pattern unlike anything hitherto recorded, 2 (4501 and 622) appear to be corynebacteria, and the remaining 6 form a homogeneous group which seems to be closely related to lactobacilli. If cell-wall composition is any guide to the classification of these strains the term *A. bovis* might well be discarded altogether, since the organisms usually given this name seem to be diverse generically and in most cases not related to microaerophilic strains of human origin.

Streptomyces, *aerobic* Actinomyces *and* Micromonospora *spp.*

These 13 strains are obviously closely related, the characteristic amino acids of their cell walls being alanine, glutamic acid, glycine and LL-diaminopimelic acid. It seems that LL-diaminopimelic acid is often accompanied by glycine in cell walls, since these two substances also occur together in propionibacteria (see Table 3), and in *Clostridium welchii* (Cummins & Harris, unpublished observations; see also Work, 1957). The single strain of propionibacteria (*Propionibacterium shermanii*, NCIB 5964) in which DL- and not LL-diaminopimelic acid occurred contained virtually no glycine. The occurrence together of DL-diaminopimelic acid and glycine has so far only been reported in two unusual strains of staphylococci (Cummins & Harris, 1956*b*).

The five strains of *Micromonospora* are distinguished by the fact that LL- and DL-diaminopimelic acid are both present. Hoare & Work (1957) have reported the presence of small amounts of DD-diaminopimelic acid, in addition to the other isomers, in whole cells of *Micromonospora*. It is not possible to say whether the DD-isomer occurs in their cell walls, as the method used in the present investigation did not separate it from the *meso*-(DL) isomer. Two of the five strains also contained the unidentified slow-moving component referred to above; its presence in strain LSTM has already been recorded by Hoare & Work (1957).

In this group of non-acid-fast aerobic actinomycetes the majority of those examined contained no detectable amounts of hexose or pentose in their cell walls. In the three cases where hexoses were present, two strains had glucose only (*Streptomyces antibioticus* 8504 and *S. olivaceus* 8238) and one had glucose + galactose (*S. aureofaciens* 8234). The cell walls of the latter also contained galactosamine, glucosamine and muramic acid.

The cell-wall compositions of the two strains of *Actinomyces graminis* and the single strain of *A. hominis* emphasize the fact that the aerobic saprophytic strains cannot satisfactorily be placed in the same genus as the microaerophilic ones, so that the generic term *Actinomyces* can be used for one or other, but not for both. Waksman & Henrici (1943) concluded that it cannot, according to the Botanical code, be used for the aerobic strains, so that if used at all it must be applied to the microaerophilic parasitic species. They proposed to call these microaerophilic strains *A. bovis* but we have indicated above why *bovis* is an unsuitable specific name for these organisms.

Finally, it may be of some interest to note that a single examination of cell walls from a purified spore suspension from *Streptomyces* sp. NCTC 7807 (Maxted strain) showed no qualitative difference in amino acid, amino sugar or

Table 3. *Cell-wall composition in* Streptomyces, *aerobic* Actinomyces, Micromonospora *and* Propionibacterium *spp.*

Name of strain	Arabinose	Rhamnose	Galactose	Glucose	Mannose	Fucose	Sugar component 'P'	Glucosamine	Galactosamine	Muramic acid	Aspartic acid	Alanine	Glutamic acid	Lysine	Glycine	Isomers of diamino-pimelic acid		Slow-moving component	Aminosuccinoyllysine
																LL	DL		
Actinomyces hominis NCTC 4525	–	–	–	–	–	–	–	+	–	+	.	+++	+++	.	+++	+++	.	.	.
A. graminis NCTC 4521 and NCTC 4728	–	–	–	–	–	–	–	+	–	+	.	+++	+++	.	+++	+++	.	.	.
Streptomyces hortensis NCTC 600	–	–	–	–	–	–	–	+	–	+	.	+++	+++	.	+++	+++	.	.	.
Streptomyces sp. (Maxted strain) NCTC 7807	–	–	–	–	–	–	–	+	–	+	.	+++	+++	.	+++	+++	.	.	.
S. antibioticus NCIB 8504	–	–	–	+	–	–	–	+	–	+	.	+++	+++	.	+++	+++	.	.	.
S. olivaceus NCIB 8238	–	–	–	+	–	–	–	+	–	+	.	+++	+++	.	+++	+++	.	.	.
S. aureofaciens NCIB 8234	–	–	++	+	Tr.	–	–	+	+	+	.	+++	+++	.	+++	+++	.	.	.
Micromonospora sp. LSTM	–	–	–	–	–	–	–	+	–	+	.	+++	+++	.	+++	++	+	+	.
Micromonospora sp. G1	–	–	–	–	–	–	–	+	–	+	.	+++	+++	.	+++	++	+	.	.
M. chalceae C	–	–	–	–	–	–	–	+	–	+	.	+++	+++	.	+++	++	+	.	.
M. chalceae S3	–	–	–	–	–	–	–	+	–	+	.	+++	+++	.	+++	++	+	+	.
M. chalceae S5	–	–	–	–	–	–	–	+	–	+	.	+++	+++	.	+++	++	+	.	.
Propionibacterium technicum NCIB 5965	–	+	++	++	Tr.	–	–	+	Tr.	+	.	+++	+++	.	+++	+++	.	.	.
P. petersonii NCIB 5962	–	–	+	+++	Tr.	–	–	+	Tr.	+	.	+++	+++	.	+++	+++	.	.	.
P. jensenii NCIB 8069	–	+	++	+++	Tr.	–	–	+	Tr.	+	.	+++	+++	.	+++	+++	.	.	.
P. pentosaceum NCIB 8070	–	–	++	++	Tr.	–	–	+	+	+	.	+++	+++	.	+++	+++	.	.	.
P. shermanii NCIB 5964	–	++	+++	±	++	–	–	+	–	+	.	+++	+++	.	.	.	+++	.	.

Tr. = trace.

sugar composition as compared with the cell walls of the mycelium. The results of Erikson (1947), however, suggest that the spores have a considerably higher content of lipid than the mycelium, and much of this may be in the spore wall.

Propionibacterium *spp.*

It was somewhat surprising to find that the amino acid pattern of the cell walls of four strains of propionibacteria was the same as that in *Streptomyces*, although the propionibacteria had a more complex pattern of sugar components. The fifth strain, *Propionibacterium shermanii* 5964, differed considerably from the others. The significance of this difference is not known and we have not examined other strains of *P. shermanii* to see whether the result is typical of this species.

Nocardia *and* Mycobacterium *spp.*

Electron micrographs of fractions from these strains prepared by the usual technique and assumed to consist mainly or entirely of cell walls showed large amounts of apparently amorphous material with few recognizable cell-wall fragments (Pl. 1, fig. 1). Chromatographic analysis of these fractions showed the presence of arabinose and galactose as characteristic sugars, and alanine, glutamic acid and diaminopimelic acid as the principal amino acids, but there were also considerable amounts of leucine, valine, threonine, serine, glycine and aspartic acid. It was noticed that oily droplets were formed during the preparation of acid hydrolysates, suggesting that much of the amorphous material visible in electron micrographs might be lipid. It has been known for some time (Anderson, Reeves & Stodola, 1937; Anderson, Creighton & Peck, 1940) that appreciable amounts of lipid remain in tubercle bacilli after extraction with neutral solvents, and that this firmly bound lipid can be released by the use of more vigorous solvents such as ethanol + ether containing 1% (v/v) conc. HCl. In view of this, an investigation was made of the effects of acid and alkaline ethanol on suspensions of nocardias and mycobacteria both before and after disintegration. It was found that while acid ethanol (0·5% (v/v) conc. HCl) had little or no effect, alkaline ethanol at 37° removed a considerable amount of material. The method finally adopted was to extract the organisms with 0·5% (w/v) KOH in ethanol for 48 hr. at 37°, after which the suspension was washed, first in neutral ethanol and then in distilled water, and the organisms disintegrated in the usual way. The insoluble fraction from these extracted suspensions was found to consist almost entirely of material having the usual appearance and dimensions of cell walls from disintegrated bacteria (see Pl. 1, fig. 2). On hydrolysis it was found that while the amounts of alanine, glutamic acid and diaminopimelic acid appeared to be unchanged, the other amino acids previously found were now absent or present only in traces. Some degradation of sugar components probably takes place in this procedure, but the main pattern was not altered. It seems obvious that alkaline ethanol removes a good deal of lipid from the insoluble fraction as prepared by disintegration in the Mickle shaker, and that this lipid may well be in the form of lipoprotein, since fewer amino acids are found after the alkaline ethanol

Table 4. *Cell-wall composition in* Nocardia *and* Mycobacterium *spp.*

Name of strain	Arabinose	Rhamnose	Galactose	Glucose	Mannose	Fucose	Sugar component 'P'	Glucosamine	Galactosamine	Muramic acid	Aspartic acid	Alanine	Glutamic acid	Lysine	Glycine	Isomers of diaminopimelic acid LL	Isomers of diaminopimelic acid DL	Slow-moving component	Aminosuccinoyllysine
Nocardia gardneri NCTC 6531	−	−	++	Tr.	−	−	−	+	+	+	·	+++	+++	·	+++	+++	·	·	·
N. caprae	+++	−	++	+	±	−	−	+	−	+	·	+++	+++	·	·	·	+++	·	·
N. asteroides NCTC 6761	+++	−	+++	++	±	−	−	+	−	+	·	+++	+++	·	·	·	+++	·	·
N. farcinica NCTC 4524	+++	−	++	−	−	−	−	+	−	+	·	+++	+++	·	·	·	+++	·	·
N. leishmanii 658	+++	−	++	+	Tr.	−	−	+	−	+	·	+++	+++	·	·	·	+++	·	·
Mycobacterium phlei NCTC 8156	+++	−	++	−	Tr.	−	−	+	−	+	·	+++	+++	·	·	·	(+++)*	·	·
M. smegmetis NCTC 8159	+++	−	++	−	Tr.	−	−	+	−	+	·	+++	+++	·	·	·	(+++)*	·	·
M. tuberculosis BCG	+++	−	++	−	−	−	−	+	−	+	·	+++	+++	·	·	·	(+++)*	·	·
M. tuberculosis H37Rv.	+++	−	++	Tr.	Tr.	−	−	+	−	+	·	+++	+++	·	·	·	(+++)*	·	·
M. tuberculosis 'C' (Human strain)	+++	−	++	−	−	−	−	+	−	+	·	+++	+++	·	·	·	+++	·	·

* D.A.P. present: assumed to be DL-isomer.

Tr. = trace.

extraction. It is not possible, however, to say from the electron micrographs whether this material is an integral part of the cell wall as it exists in the living organism, or whether it represents adherent remains of cytoplasmic lipid.

The presence of arabinose and galactose together with alanine, glutamic acid and *meso*-diaminopimelic acid suggests most strongly that both the nocardias and the mycobacteria are closely related to the corynebacteria, in which we had previously found this pattern of cell-wall components (Cummins & Harris, 1956*a*). It has long been recognized on morphological grounds that such a relationship exists, and many suggested classifications of the actinomycetes have included *Mycobacterium* and less frequently *Corynebacterium* as constituent genera of the order Actinomycetales. The resemblances between their cell-wall compositions is so striking however, that one is forced to the conclusion that taxonomically nocardias, mycobacteria and corynebacteria should stand or fall together, so far as inclusion in any larger grouping is concerned.

It has already been noted that there is a clear distinction between the cell walls of *Streptomyces* and *Nocardia* (Cummins & Harris, 1956*a*; Romano & Sohler, 1956), and this is borne out in an interesting way by the strain *Nocardia gardneri* NCTC 6531. The cell-wall composition of this organism is quite different from that of the other strains of *Nocardia* examined (see Table 4). It is, however, virtually identical with that of *Streptomyces aureofaciens* NCIB 8234 and there seems little doubt that the strain *N. gardneri* NCTC 6531 should be reclassified as a *Streptomyces*.

The cell walls of *Nocardia* spp. are distinguished from those of *Streptomyces* spp. by the fact that they contain arabinose, and also because DL-diaminopimelic acid is present instead of the LL-isomer found in *Streptomyces*. Hoare & Work (1957) have already shown that these genera can be separated by an examination of the type of diaminopimelic acid present in whole cell-hydrolysates, and we have found in a small number of strains (Cummins & Harris, unpublished) that the presence or absence of arabinose in hydrolysates of intact organisms can also be used to identify them. This would seem to be a useful additional method for distinguishing members of these two genera in cases of doubt, without the necessity for a full cell-wall analysis. The final identification of atypical strains of *Nocardia* or *Streptomyces* by morphology or fermentation tests may be somewhat difficult (see Gordon & Mihm, 1957).

DISCUSSION

Relationship of the actinomycetes to bacteria and fungi. On account of their morphology, particularly in the case of *Streptomyces* and *Micromonospora*, it has often been suggested that the actinomycetes form a group intermediate between the bacteria and the fungi. Most of the evidence, however (for example the absence of true nuclei and the size of the mycelium) points to a far closer relationship to the Eubacteriales than to the Eumycetes (see Waksman, 1957). Avery & Blank (1954) were unable to detect cellulose or chitin in strains of *Actinomyces*, *Nocardia*, *Streptomyces* or *Micromonospora*, and concluded that they had nothing in common, chemically, with the true fungi. The present results show that the cell walls of the actinomycetes are complexes of sugars, amino sugars and a few amino acids (usually three or four) and are thus identical in general structure with cell-wall fractions from Gram-positive bacteria (Salton, 1953; Cummins & Harris, 1956*a*, *b*). Two particularly characteristic features are the presence of the substituted hexosamine muramic acid, and the restricted number of amino acids, among which either diaminopimelic acid or lysine, but not both, is a major component.

Table 5. *Composition of walls of various fungal mycelia*

	Sugars and amino sugars							
	Arabinose	Rhamnose	Galactose	Glucose	Mannose	Glucosamine	Galactosamine	Muramic acid
Penicillium species	–	–	++	+++	+	++++	–	–
Aspergillus species	–	–	++	+++	+	++++	–	–
Rhizopus stolonifer 26441	–	–	–	–	–	++++	–	–
Mucor mucedo 42844	–	–	–	–	–	++++	–	–
Microsporum gypseum	–	–	–	f.Tr.	f.Tr.	++++	–	–
Tricophyton mentagrophytes	–	–	–	Tr.	–	++++	–	–
Epidermophyton floccosum	–	–	–	++	–	++++	–	–

Tr. = trace. f.Tr. = feint trace.

As a comparison we have examined by the same technique hydrolysates of mycelial walls from a limited number of species of fungi, and the results are recorded in Table 5. Apart from traces of the amino acids most commonly found in proteins, which probably come in this case from a slight degree of cytoplasmic contamination, the walls of these fungi consist entirely of carbohydrate. There is, moreover, no trace of muramic acid, although large amounts of glucosamine are present. These results seem to indicate a fundamental biochemical distinction between fungi and actinomycetes. The substance muramic acid in particular seems to be a characteristic bacterial product in both Gram-positive and Gram-negative species (Cummins & Harris, 1956*a*, *b*, and unpublished observations), and its complete absence from these fungi is striking.

It may also be noted in passing that there is a difference in composition between the walls of the 2 strains of *Aspergillus* and *Penicillium* examined, and those of *Mucor* and *Rhizopus*, since the latter consisted entirely of glucosamine while the former contained appreciable amounts of hexoses in addition. This difference is interesting since it has been previously suggested that cell-wall composition in fungi may have taxonomic significance (see Frey, 1950).

The classification of the actinomycetes. If the organisms which we have examined are classified solely on cell-wall composition, a tentative scheme can be drawn up which is outlined in Table 6. In this scheme the genera proposed are those of Waksman & Henrici (1943) except that it is suggested that strains of animal origin should not be included in *Actinomyces* at least until their position has been clarified by further work. One feature of the proposed classification is that *Nocardia* is separated from *Actinomyces* and placed instead in the family Mycobacteriaceae together with *Mycobacterium* and *Corynebacterium.*

Table 6. *Suggested classification of actinomycetes, based on cell-wall composition*

		Characteristic cell-wall components	
Family	Genus	Sugars	Amino acids
Mycobacteriaceae	*Nocardia* *Mycobacterium* *Corynebacterium*	Arabinose, galactose	Alanine, glutamic acid, DL-diaminopimelic acid
Actinomycetaceae	*Actinomyces*	Galactose	Alanine, glutamic acid, lysine
Streptomycetaceae	*Streptomyces* *Micromonospora* (*Propionibacterium*)	No characteristic sugar	Alanine, glutamic acid, glycine, LL-diaminopimelic acid*

* In *Micromonospora* DL-diaminopimelic acid is present in addition.

This arrangement is the same as that suggested by Bisset & Moore (1949) on the basis of cell morphology. There seems to be every reason to regard *Mycobacteria*, *Nocardia* and *Corynebacteria* as closely related and the fact that all have the same characteristic cell-wall composition, distinguished particularly by the pentose arabinose, is an additional indication that they should be classified together.

A further property possessed by many species of *Nocardia* is that of acid-fastness. This is a difficult character to use in classification, since it is dependent to a large extent on the age of the culture, the medium and the length of time since the strain was originally isolated. It seems most likely that all strains of *Nocardia* are at least potentially acid-fast, so that the possession of this character is significant but its absence is not. The distinction between the two genera *Nocardia* and *Mycobacterium* is very blurred, and organisms such as *M. phlei* may show a branched mycelium at some stage of growth, while cultures of *Nocardia asteriodes* may at times be composed almost entirely of acid-fast rods. The distinction between *Corynebacterium* and *Nocardia* is more clear-cut in this respect, but at least some corynebacteria can be acid-fast (e.g. *Corynebacterium equi*; see Jensen, 1953, p. 82).

As far as other genera in the proposed classification are concerned, little comment is needed. Almost all authorities are agreed that *Streptomyces* and *Micromonospora* should be placed in the same family, and the results of cell-wall analysis support this. A more detailed investigation of a series of strains of propionibacteria will be needed, however, before the relationship between them and the streptomyces can be definitely established. We have therefore included *Propionibacterium* in Table 6, but placed it in brackets to indicate its rather doubtful position in the Streptomycetaceae. Hungate (1946) described an anaerobic organism *Micromonospora propionici* which exhibited a characteristic propionic acid fermentation, so that there may well be a relationship between the two groups. In the case of *Actinomyces*, it seems best to leave this for the moment as a genus containing a single species, *A. israelii*. Cell-wall composition gives no indication of any close relationship between *Actinomyces* and *Corynebacterium*, nor between *Actinomyces* and *Lactobacillus bifidus* (see Cummins *et al*. 1957); the products of fermentation of glucose are also different in all three groups (Pine, 1956; Pine & Howell, 1956).

A final question which seems to us to need discussion is whether it is desirable to have two separate orders, Actinomycetales and Eubacteriales. These are usually distinguished on the grounds that the bacteria proper do not show mycelium formation and branching, while the actinomycetes do. This is certainly true of strains of *Streptomyces* and *Micromonospora* in which a non-fragmenting branched mycelium is a characteristic feature. On the other hand, corynebacteria do not usually exhibit either branching or a mycelial type of growth, and yet there is every reason to suppose that they are very closely related to the nocardias in which both of these characters are present. It has already been pointed out that the general pattern of cell-wall components in actinomycetes is the same as that in Gram-positive bacteria. Although the subject is too large for a full discussion here, it seems to us that a good case can be made for abandoning the order Actinomycetales, and for classifying actinomycetes together with the bacteria in the Eubacteriales. Many organisms will produce filamentous forms under certain conditions, for example, in magnesium-deficient media (Webb, 1948, 1949). The ability to grow in the form of branched filaments could have evolved independently in several different groups, in which case it would be unwise to use it too rigidly as a differential criterion, particularly if a phylogenetic system of classification is ultimately to be adopted.

We are grateful to Professor C. F. Barwell and Dr S. T. Cowan for advice and criticism during the preparation of the manuscript, to Miss Sylvia Start for much assistance with the chromatography, to Miss Olivia Glendenning for help in the preparation of cell-wall fractions and to Dr R. C. Valentine of the National Institute for Medical Research, Mill Hill, for the electron micrographs. We would also like to thank all those who sent cultures for investigation.

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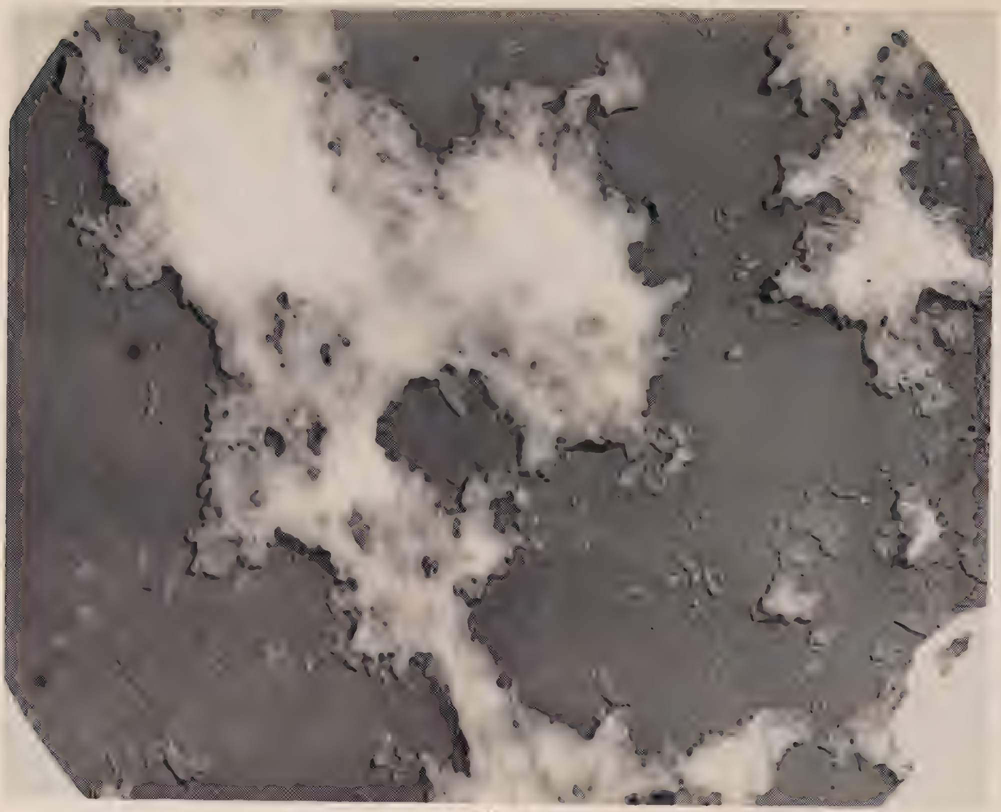

Fig. 1

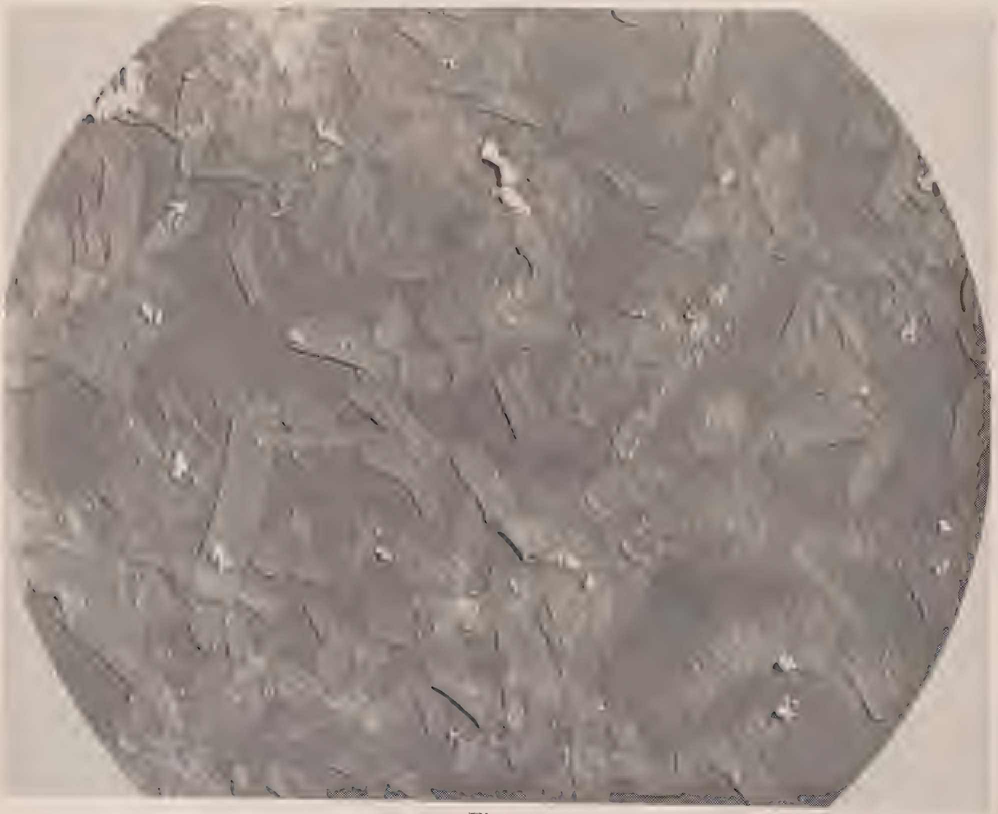

Fig. 2

C. S. Cummins & H. Harris—Cell wall and taxonomy of actinomycetales. Plate 1

(*Facing p.* 189)

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EXPLANATION OF PLATE

Magnification in both photographs *c.* ×12,000.

Fig. 1. Cell-wall fraction from suspension of *Mycobacterium tuberculosis* strain C (human) which had been extracted with neutral solvents before disintegration.

Fig. 2. Cell-wall fraction from suspension of *M. tuberculosis* strain C extracted with alkaline ethanol before disintegration.

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Artificial Cultivation of a Red-Pigmented Marine Blue-Green Alga, *Phormidium persicinum*

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SUMMARY: *Phormidium persicinum* requires light intensities <300 foot-candles and grows at 10°–33°. The medium developed for *P. persicinum* is representative of the early types of artificial media having a relatively high concentration of chelator and, necessarily, of trace metals. Other media, varying widely in trace metal: chelator ratios and concentrations allow growth of *P. persicinum*. Tris 2-amino-2-hydroxymethylpropane-1:3-diol is a suitable pH buffer; its antagonism of potassium utilization was demonstrated and was amply overcome by the addition of 200 mg. K/l. to the medium. Inorganic and organic phosphates were utilized and were required at relatively low concentrations. *P. persicinum* utilized NO_3^- and NH_4^+ but not atmospheric nitrogen. $NaNO_3$ was the most satisfactory inorganic nitrogen source but the cultures bleached after prolonged growth. Asparagine (0·2–0·8 g./l.) maintained the normal purple pigmentation over a longer period; it served as a non-toxic slowly-utilized reservoir of available N. Vitamin B_{12} is the only essential vitamin and is needed at a concentration (100 mμg./l.) which is higher than for most algae. All the cobalamins including factor B, but not methionine or deoxyribosides, can substitute for vitamin B_{12}. The physiological and ecological resemblances of *P. persicinum* to red or violet blue-green algae growing in deep water are discussed.

The nutrition and other aspects of the physiology of the blue-green pigmented members of the Cyanophyceae have been much studied but little has been done with the red-pigmented species, which, according to ecological observations, colonize and prefer deeper water layers.

Phormidium persicinum was isolated from a bottle of Wood's Hole sea water which had been stored in dim fluorescent light for 6–8 months. The growth appeared as small purple tufts at the bottom and on the walls of the bottle. Fragments were placed on the centre of agar plates of Föyn's 'Erdschreiber' sea water (Föyn, 1934) to which had been added 1000 units penicillin/ml. and 25μg. dihydrostreptomycin/ml. *P. persicinum* in liquid media withstands 20μg. dihydrostreptomycin/ml. for a month with no bleaching; other blue-green algae appear to be much more sensitive to streptomycin. Heavy bacterial growth was suppressed by the antibiotics and the filaments grew rapidly outward, leaving most bacteria behind. Two transfers of filaments from the outer zone were necessary to establish a bacteria-free culture (by cutting a block of agar with filaments from the outer zone and inverting on a new plate without antibiotics). Cultures were at first maintained on Föyn's 'Erdschreiber' agar slopes, and later in medium ASW III (Table 1).

In all but the earliest experiments, an all-glass technique described by Hutner, Provasoli & Filfus (1953) was used. Experimental cultures were grown in 10 ml. of liquid media in 25 ml. Erlenmeyer flasks with glass caps.

To prepare uniform inocula from the thick filamentous mat, cultures were grown with sterile glass beads and shaken at intervals as suggested by Fogg (1942); the alga then grows in more or less separate filaments. In later experiments, to obtain comparative values of growth within each experiment and from one experiment to another, cultures were ground for 5–10 min. in a 'Virtis' micro-homogenizer and the optical density measured with a Densichron (W. M. Welch Sci. Co., Chicago).

Table 1. *Sea-water medium for maintenance of* Phormidium persicinum

Medium ASW III

Sea water	100 ml.	NaH glutamate	0·05 g.
KNO_3	0·02 g.	Glycine	0·05 g.
K_2HPO_4	0·002 g.	'1:20 liver'*	0·001 g.
Fe (as chloride)	0·01 mg.	Vitamin Mixture 8†	0·1 ml.
Soil extract	4 ml.	pH 7·5	
Mn (as chloride)	0·04 mg.		

* An aqueous extract of liver: Wilson and Co. Chicago, U.S.A.

† Cowperthwaite, Weber, Packer & Hutner (1953).

RESULTS

Physical factors which affect growth of Phormidium persicinum

We soon saw that illumination good for other photosynthetic organisms did not suit *Phormidium persicinum*. Light intensities >300 ft.c. hampered pigment formation and growth; the optimal intensities lay between 100 and 200 ft.c. of white fluorescent light. *P. persicinum* grows at temperatures up to 33°; experiments were normally carried out at 20°–25° and the stock cultures were kept at 10°–15° in dim light. In stationary liquid cultures, *P. persicinum* tended to form a thick mat on the surface; there was usually also some submerged growth, especially in test tubes. In one experiment, aeration for 17 days did not speed growth or increase the yield.

Mineral nutrition

The first chemically-defined medium to support good growth contained organic constituents (amino acids, purines and pyrimidines) later found inessential. The present medium (Table 2) clarified several points concerning the construction of chemically-defined media for marine organisms (Provasoli, McLaughlin & Droop, 1957). Ca and Mg concentrations are widely interchangeable, as with many other marine organisms (Provasoli, McLaughlin & Pintner, 1954). With a concentration of Mg corresponding to that in sea water (1·2 g./l.), the Ca concentration can be decreased to 10 mg./l. (1/40 sea-water concentration); Mg above sea-water concentration inhibits. With Ca at 300 mg./l. (3/4 sea water), Mg can be decreased to 50–100 mg./l. Calcium at 80 mg./l. and Mg at 600–700 mg./l. are optimal in a medium containing 200 mg. ethylenediaminetetraacetic acid (EDTA)/l. at pH 7·0–7·2. Similar Ca + Mg combinations can be employed with different concentrations of trace-metal + chelator mixtures.

Concentrations of NaCl >3% or <1·5% (w/v) decrease growth. NaCl cannot be substituted osmotically, even partly, by pentaerythritol which seems toxic at concentrations as low as 0·5 g./l.

Potassium is needed in low concentration (5·0 mg./l.). Since most media become alkaline during growth, buffering to maintain a constant pH value is important in order to prevent a damaging shift in metal balance. Of the buffers tried, tris was the most satisfactory. However tris (buffer) antagonized K utilization by bacteria (MacLeod & Onofrey, 1954). This also holds for *Phormidium persicinum*: with 1 g. tris (buffer)/l., which buffers adequately, the requirement for K was raised to 40–50 mg./l. Our present media have ample K (400 mg. KCl/l. = 200 mg. K/l.) thus avoiding antagonism by tris (buffer). With suitable adjustment of the trace metals, *P. persicinum* can be grown between pH 6·0 and pH 8·0.

Table 2. *Defined medium for* Phormidium persicinum

EDTA	20 mg.	$MgSO_4.7H_2O$	0·5 g.
NaCl	2·5 g.	$NaNO_3$	10 mg.
Metal mix*	0·4 ml.	DL-Asparagine	20 mg.
Fe (as chloride)	0·4 mg.	K_3PO_4	5 mg.
KCl	40 mg.	Vitamin B_{12}	0·2 μg.
Ca (as chloride)	8 mg.	Tris	100 mg.

Distilled water to 100 ml. pH adjusted to 7·0

* Metal mix. 100 ml. of the mixture contains the following concentrations of the metals (mg.; calculated as chlorides): Mn 4; Zn 4; Cu 0·1; Co 0·05; Fe 0·2; citric acid.H_2O 3.

Phormidium persicinum can utilize inorganic and organic phosphates; 0·3 mg. K_3PO_4/l. is the minimal concentration for appreciable growth and it is tolerated up to at least 300 mg./l.; K glycerophosphate is utilized from 0·3 to 10 mg./l.; 100 mg./l. is toxic. Guanylic, cytidylic, and yeast adenylic acids are also utilizable as phosphorus sources from 1 to 100 mg./l., with optimal growth at 10 mg./l. These values are much lower than those required by *P. autumnale*, a fresh-water species now being studied.

Atmospheric nitrogen is not utilized by *Phormidium persicinum*. Fogg & Wolfe (1954) reported that many blue-green algae fix nitrogen, others not. They suggested that this inability may result from lack of molybdenum. The addition of 0·2 mg. Mo/l. to the *P. persicinum* medium did not permit utilization of N_2 nor was growth with nitrate or ammonia enhanced. Molybdenum is apparently amply supplied as an impurity in the reagent-quality salts of the medium. Both NO_3^- and NH_4^+ are good N sources and, as Kinne-Diettrich (1955) reported for two marine species (*Lyngbya confervoides*, *L. maiscula*), NO_3^- is better than NH_4^+. Glycine is poor, and lysine, histidine, glutamic acid and aspartic acid are inert as N sources. Asparagine is utilized, and nitrate + asparagine gives better growth than either alone. Biotin (up to 1 mg./l.) does not increase asparagine utilization.

No carbon sources tested affected total growth (i.e. they seemed not to be utilized) and they did not permit growth in darkness, yet they seemed to influence the permanence of the normal purple colour.* Acetate, glycerol, mannitol, succinate, fumarate and ethanol as single added C sources gave orange-yellow to dirty yellow-brown cultures; glycine, alanine and asparagine maintained normal pigmentation. Cultures with NO_3^- (100–200 mg. $NaNO_3$/l.) as the sole N source turned pink, then yellowish and finally bleached after a month or so. With asparagine 200–800 mg./l. added to the NO_3^- medium, the purple coloration persisted for at least 3 months; glycine and alanine acted in the same way but the cultures faded earlier; this did not depend on the utilization as a carbon source of the deaminated molecule, for aspartic acid added to the medium containing NO_3^- or NH_4^+ neither increased growth nor favoured persistence of pigmentation.

The medium developed for *Phormidium persicinum* represents one of the earlier types of artificial marine media which contained a relatively high concentration (200 mg./l.), of ethylenediaminetetraacetic acid (EDTA) and a correspondingly higher content of trace metals. This ratio is important, as is total concentration of minor elements necessary to compensate for the binding action of EDTA. A single heavy metal, e.g. Fe or Zn, is tolerated in higher concentration when the other trace elements are at a higher concentration also. This might explain why mixtures of chelator and trace metals like that embodied in the medium ASP_7 of Table 3 are non-toxic in a wide range of concentrations. Total salinity and the Ca:Mg and trace metal: chelator ratios and concentrations can differ quite widely as indicated by the ability of *P. persicinum* to grow in diverse artificial media. In the earlier, presumably unbalanced, media the addition of guanine or other purines and pyrimidines was stimulatory. This effect was also observed during the development of good basal media for other fresh-water and marine organisms, and can be ascribed to interactions and impurities.

Table 3. *Marine medium* ASP_7; (*Provasoli, unpublished*)

NaCl	2·5 g.	PI metal mixture*	3 ml.
$MgSO_4 . 7H_2O$	0·9 g.	NTA (nitrilotriacetic acid)	7 mg.
Ca (as chloride)	30 mg.	Tris	0·1 g.
KCl	0·07 g.	Vitamin B_{12}	0·1 μg.
$NaNO_3$	5 mg.	Vitamin mixture†	1 ml.
K_2 glycerophosphate	2 mg.	H_2O to	100 ml.
$Na_2SiO_3 . 9H_2O$	7 mg.	pH 7·8–8·0	

* *PI metal mixture.* 1 ml. contains (mg.): Na_2EDTA 1; Fe (as chloride) 0·01; Boron (as H_3BO_3) 0·2; Mn (as chloride) 0·04; Zn (as chloride) 0·005; Cu (as chloride) 0·00004; Co (as chloride) 0·0001.

† See Table 1. Not essential for *Phormidium persicinum.*

Growth factors

The mineral requirements of *Phormidium persicinum* were studied in media containing a mixture of the B vitamins (Cowperthwaite, Weber, Packer &

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* The colour values given in the text are followed by the corresponding standard colour descriptions of Ridgway (1912): purple = between 71 K (Bordeaux) and 69 K (Pansy Purple); pink = 1 f (Venetian Pink); orange-yellow = 15 b (Ochraceous Buff); dirty yellow-brown = 17 K (Dresden Brown); yellowish = 21 b (Amber Yellow); red-brown = 7 m (Liver Brown).

Hutner, 1953). During the dissection of this mixture to find the vitamins responsible for growth, thiamine seemed needed (Provasoli & Pintner, 1954). This effect disappeared following complete depletion of the organisms by serial transfers of washed inoculum; only vitamin B_{12} was then required. The need for cyanocobalamin was high in comparison with that of other algae, namely 0·1 μg./l. for full growth. All the other cobalamins, even factor B (the non-nucleotide moiety of the cobalamins) substituted for intact vitamin B_{12} (Table 4). This lack of specificity is similar to that for *Escherichia coli* 113–3, *Amphora perpusilla* (McLaughlin & Provasoli, unpublished), and *Skeletonema costatum* (Droop, 1955). *Phormidium persicinum* seems unable to replace vitamin B_{12} with methionine (as can *Escherichia coli*) or with deoxyribonucleotides (as can *Lactobacillus leichmannii* and *L. lactis*). This broad specificity may constitute a significant ecological advantage (Provasoli, 1957).

Table 4. *Growth response of* Phormidium persicinum *to cobalamins*

The relative amounts of growth are expressed in optical density values.

	Concentration of factor added (mμg./l.)		
	10	100	1000
	optical density values		
Cobalamin tested			
Vitamin B_{12}	0·24	0·50	0·74
Pseudo B_{12}	0·36	0·49	0·62
Factor A	0·31	0·48	0·51
Factor B	0·23	0·43	0·64
Factor H	0·36	0·43	0·49
Factor I	0·32	0·46	0·63
Benziminazole analogue	0·43	0·44	0·47
5:6-Dichlorobenziminazole analogue	0·40	0·41	0·47
Control (no vitamin B_{12})	0·04	0·04	0·04

DISCUSSION

Wille (1922) found that *Phormidium persicinum* is abundant at depths of 20 m. on the south coast of Norway. Many records show that the blue-green algae inhabiting deep waters are red or violet; numerous red algae also thrive in the same locale. The ability of these algae to grow in deep waters is due to their accessory pigments (*c*- or *r*-phycoerythrin) which enables them to utilize blue-green light, the light which penetrates deeper into the waters. None the less such ability should not prevent them from growing in shallower waters where the entire spectrum is offered them, and other factors, such as light intensity (low in deep waters) should become significant for the species which are consistently found only in deeper waters. Indeed, *P. persicinum* is hampered by light intensities above 300 ft.c. and prefers intensities between 100 and 200 ft.c. Since the cultures were grown under fluorescent lights (rich in blue-green light) our conditions may resemble the natural habitat of the species. *P. persicinum* appears, then, to be a true shade form admirably fit to colonize its ecological niche. Fritsch has already postulated that the blue-green algae may be divided into light- and shade-loving species (Fritsch, 1945, p. 785).

Other red-pigmented blue-green algae may have similar light requirements: Feldman (1932) collected from draggings at Banyuls (Pyrénées Orientales, France) several species of red-pigmented Cyanophyceae, which were living epiphytically at a depth of 30 m., among them *Trichodesmium erythraeum*. *T. erythraeum* had been considered a pelagic species because of its periodic surface blooms which are responsible for the naming of the Red Sea and the Vermillion Sea (Gulf of California). Feldman postulated that *T. erythraeum* may actually grow far down and, under certain conditions, become detached, abandon its usual habitat and float at the surface; our observations on the light requirements of *Phormidium persicinum*, which, like *Trichodesmium erythraeum*, is an Oscillatoriacea, support Feldman's conclusions.

Some red-pigmented blue-green algae and red algae change colour (so-called colour adaptation) under different light conditions. Light of different wavelengths induces different pigmentation from red to orange, green and blue-green (Boresch, 1921; Dangeard, 1911); however, Kylin (1937) and Harder (1922) have shown that light intensity (which was not controlled in Boresch's experiments) also influences the type of pigmentation. Even so Kylin supports the view of Boresch that colour adaptation is only possible in species possessing both phycocyanin and phycoerythrin and results from variations in the ratio of these two pigments; Boresch had not found any relation between quantity of chlorophyll and wavelength of incident-light. Improved chromatographic techniques offer the opportunity to resume the study of selective production of phycobilins in blue-green algae.

Colour changes are also induced by nutritional deficiencies or toxicities. Such changes have been observed in *Phormidium persicinum* and may be specific because they were observed under uniform light conditions and because this species has only *c*-phycoerythrin and no phycocyanin (Haxo, O'hEocha & Norris, 1955). The obvious differences in pigmentation induced us to record the colour of the mats of filaments; the purple colour was taken as normal because this is the usual colour in nature and because it generally accompanies maximum growth. Whenever concentrations of nutrients or light conditions were unfavourable for growth, the red brownish colour prevailed, followed by the appearance of green patches, yellow-beige discoloration and, finally, bleaching. The pigment sometimes leached into the medium after two months: this happened more often in media containing NH_4^+ than with NO_3^-; the pH value remained constant. Most brackish or marine organisms are sensitive to NH_4^+ in the alkaline range (Provasoli *et al.* 1957) and the chrysomonad *Prymnesium parvum* actually lyses (Shilo & Shilo, 1953). It may be that the loss of pigment reflects a change in permeability in old cultures of *P. persicinum*, even though NH_4^+ does not hamper growth up to 0·5 g. NH_4Cl/l.

As noted, colour changes were most conspicuous with certain carbon sources. Asparagine, and to a lesser degree glycine, promoted a brilliant purple colour and prevented bleaching of old cultures; the higher the concentration of asparagine, the greater the duration and intensity of coloration. This effect might be due to several causes, among them impurities in the asparagine but, different samples, one of them synthetic (California Foundation for Biochemical Research), gave similar results. The pH value of the cultures with or without asparagine remained at pH 7·7 throughout the period of growth; this observation appears to eliminate a gross change in availability of trace metals as being responsible. Addition of a mixture of Sr, Li, I, Br, B, Rb, of various mixtures of trace metals, or of single heavy-metal salts, to low concentrations of asparagine (200 mg./l.) did not elicit the lasting pigmentation obtained with higher concentrations of asparagine (600–800 mg./l.). Furthermore, *Phormidium persicinum* grew in many chemically-defined media developed for other marine protists (Provasoli *et al.* 1957) and in medium ASP_7 (Table 3). At least several serial transfers could be made in these media which differed widely in respect to the content of chelator and trace metals, and to the chelator : trace metal ratio, which lessened the possibility of a deficiency in trace metals influencing pigmentation. Here again addition of asparagine resulted in prolonged pigmentation, suggesting that asparagine functioned primarily as an N source. Completely bleached old cultures grown with $NaNO_3$ regained their purple colour in a few days after more $NaNO_3$ was added. The effect of N-deficiency on the coloration of several Rhodophyceae was described by Haxo & Strout (1950) who observed uniform reddening within 24 hr. after addition of KNO_3. Since increased concentrations of $NaNO_3$ used alone (up to 1 g./l.) increased the duration of pigmentation but did not equal that afforded by 100 mg. $NaNO_3$ + 600–800 mg. DL-asparagine/l., it seems likely that asparagine is utilized slowly and, being non-toxic even at high concentrations, provides a more lasting N reservoir and so permits growth during a longer period. It is possible that amino acids such as asparagine, alanine, etc., which favour pigmentation of *P. persicinum*, supplement the scarce nitrates of warm seas and might even be important sources of N for algae growing in the depths, which implies that ability to utilize organic N is a significant ecological advantage. *P. persicinum* was found by Wille growing on empty bryozoan shells attached to *Laminaria saccharina*—an environment presumably rich in products of bacterial metabolism. *Trichodesmium erythraeum* was found living also epiphytically at similar depths by Feldmann (1932), which suggests that these two species which share a common environment and may have similar light requirements may have common nutritional characteristics. These considerations increase the likelihood that *P. persicinum* may serve as a guide in cultivating other ecologically important marine blue-green algae.

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Lysogenicity and Immunity to Bacillus Phage W

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SUMMARY: Cultures of strain W of *Bacillus cereus* sometimes contained a phage, Wα, which could form plaques on strain W itself. Phage Wα was found to be a virulent mutant of a temperate phage, Wβ, with which strain W was lysogenic. Another non-lysogenizing mutant, Wγ, was also isolated which, unlike α, did not form plaques on strain W. The genetic determinants of α, β and γ are probably allelic. Neither phage γ nor phage β formed plaques on bacterial strains lysogenic with β, but these strains were not completely immune, even to the temperate phage. This finding shows that lysogenicity does not invariably confer complete immunity to the carried phage. The colonial appearance of a strain was altered after lysogenization in a way suggesting that the alteration was due to its incomplete immunity to the temperate phage produced during growth.

Lysogenic strains of bacteria are usually immune to the phage they produce and the immunity is conferred on them by the prophage (Lwoff, 1953; Bertani, 1958). The phages produced by lysogenic strains are temperate (by definition capable of lysogenizing), but temperate phages can give rise by mutation to virulent phages (by definition incapable of lysogenizing; Jacob, Lwoff, Siminovitch & Wollman, 1953). In general, lysogenic strains are immune to the commoner virulent mutants as well as to the temperate phage, and thus do not manifest any cultural abnormality to suggest that they produce a phage. A strain of *Bacillus cereus*, named W, attracted attention because platings from old broth cultures yielded bacterial growth which was interrupted by patches of partial clearing. A phage was isolated from these patches which produced plaques on strain W. It was easily shown that the cultures were not merely cultures contaminated with phage, and thus it appeared that strain W produced a phage to which it was itself susceptible. The most probable explanation for the phenomenon was that the phage was a mutant of a temperate phage with which strain W was lysogenic. The phage, which has been called phage Wα, also attacked strains of *Bacillus anthracis* and a small proportion of strains of *B. cereus* (McCloy, 1951). It did not lysogenize sensitive strains. Subsequently, other phages were isolated from *B. cereus* strain W which did not form plaques on it. Filtrates of cultures of strain W of any age contained a phage, designated Wβ, which attacked all strains which were sensitive to phage α except strain W. Phage β could lysogenize, and the lysogenic derivatives of sensitive bacterial strains were, like strain W, grossly susceptible to phage α but not to phage β. A third phage, named Wγ, found in exponentially growing cultures of strain W, did not lysogenize, and attacked strains sensitive to phage β. Phage γ did not form plaques on strains lysogenized by β. Bacteria artificially lysogenized by phage β gave rise spontaneously to phages which resembled α and γ in all characters tested, and which were therefore assumed to be the same as the phages α and γ directly isolated from strain W.

The three phages α, β and γ are identical in serological and other characters (McCloy, to be published). The present paper discusses the relationship between them, and their behaviour in relation to the phenomena of lysogenicity and the immunity which it confers. Phage β had the characters of a temperate, that is a potentially lysogenizing, phage, and phages α and γ were virulent, that is unable to lysogenize. Phage α was differentiated from phage γ by its ability to form plaques on the lysogenic strains; as will be seen, however, phages γ and β were not quite without action on the lysogenic strains.

All single colony isolates of *Bacillus cereus* strain W which were tested yielded the three phages, α, β and γ. Hence, each cell of strain W was presumed to carry the precursor of each phage. Since lysogenization of sensitive strains with phage β always yielded cultures from which phages α and γ could be obtained, the three phages evidently had a common genetic origin; this conclusion was also supported by their identity in serological and other characters. The precursor in the naturally lysogenic strain W was probably β because, of the three, β was the only temperate phage in relation to the sensitive indicator strains used, and because in strain W, as in the strains artificially lysogenized with β, phage β was produced regularly, whereas α and γ appeared more rarely, as if their appearance were a consequence of mutation.

METHODS

Bacteria. The lysogenic strain of *Bacillus cereus*, strain W, had the characters of the species *B. cereus* (Smith, Gordon & Clark, 1952; Knight & Proom, 1950; Chu, 1949 and personal communication). The strain used in most of the experiments as indicator and propagating strain for the phage produced by strain W was an atypical strain of *B. anthracis*, strain Davis, since confluent growth of this strain was very much smoother than that of typical strains of *B. anthracis*, and plaques were easily seen in lawns produced either by flooding plates with bacterial suspensions or by the agar layer method. Strain Davis was identified as a strain of *B. anthracis* by Dr H. P. Chu (personal communication), but it was atypical in that it was of low virulence for mice, did not form spores, and its cells were less than $0{\cdot}9\,\mu$. in diameter. It grew in broth as a mixture of short chains and filaments, giving a uniform turbidity. No means were found of making it grow in elements more uniform in length. It formed small colonies of rather rough appearance on nutrient agar incubated in air (Pl. 1, fig. 3), but the growth could be emulsified fairly easily, in contrast to the growth of typical *B. anthracis*. It had retained the ability to form capsules, for it formed mucoid colonies on serum agar incubated in air with added CO_2 (Sterne, 1937; Chu, 1952). Other strains used as indicators and propagating strains for the phage were an avirulent asporogenous strain of *B. anthracis* called Lindsay, which formed almost smooth colonies, and three typical virulent strains of *B. anthracis*, strains 2160, 1444 and H_3. Variants of strain 2160 which formed colonies smoother than those of the parent strain, and which were asporogenous, were isolated by incubating for some days cultures of strain 2160 in 100 ml. volumes of broth to which 0·025 % (w/v) $CaCl_2$ had been added (Bordet & Reneaux, 1930; Reneaux, 1952); one such variant, called 2160S, was used in some experiments. One strain of *B. cereus*, NCTC 1651, which was sensitive to the phage, was also used. None of the strains of *B. anthracis* could be shown to be lysogenic. Usually only the strain names will be used in this paper except where the species (*anthracis* or *cereus*) of the strain is relevant to the subject.

Measurements of bacterial turbidity were made with an EEL nephelometer (Evans Electroselenium Ltd, Harlow, Essex).

Media. The fluid media used in most of the experiments were Hartley tryptic digest broth, and peptone meat-extract (Lemco) broth. The solid medium consisted (as %, w/v) of: 1, peptone (Evans); 1, meat extract (Lemco); 1, NaCl; 1·2, New Zealand agar (Davis), and was supplemented with 0·5 % (w/v) liver extract (Oxoid). On this medium without the liver-extract supplements, lawns of strain Davis were thin, and the plaques produced by the temperate phage β were clear and could not be distinguished from those produced by phages α and γ. It was found, however, that addition of liver extract gave thicker lawns, and that the plaques of phage β then contained bacterial growth and could now easily be distinguished from plaques of phages α and γ which were still clear.

Phage stocks. These were made by growing the phages on sensitive strains in broth. Stocks of the phages grown on strain Davis were also made by the agar layer method (Swanstrom & Adams, 1951), since by this method higher titres were obtained. Lysates were filtered through sintered-glass or Chamberland filters. With preparations grown on strain Davis, titres of 1×10^9 to 5×10^9 particles/ml. were obtained by the broth method, and titres of 1×10^{10} to 5×10^{10} particles/ml., and occasionally as high as 2×10^{11} particles/ml. were obtained with phages α and γ by the agar layer method.

Artificially lysogenic strains. These were made by exposing sensitive strains to the phage on solid medium; the resulting bacterial growth was subcultured, and an isolated colony entire in outline was tested for the presence of phage. Three or four successive single-colony isolations were made from satisfactory colonies before the bacterial strain was used. Lysogenic strains have been named in the conventional way with the name of the phage in brackets following the name of the bacterial strain, for example Davis (β).

Phage βLC. In the earlier experiments, bacterial strains lysogenic with β were the only available differential indicators for phage α, and they were unsatisfactory indicators in that they themselves could produce phage α, and fairly frequently showed spontaneous phage α plaques. However, in the course of the work, a variant of phage β was obtained which was not observed to give rise by mutation to phage α; with this phage very much more satisfactory differential indicator strains could be made for detection and titration of phage α. This variant phage, named βLC, was derived as a spontaneous mutant of a plaque-type mutant arising when irradiated phage β was plated with irradiated bacteria of strain Davis (Weigle, 1953). The genetic differences between phage β and phage βLC have not been investigated, but phage βLC evidently differs from phage α at more than one locus, for mixed infection of strain Davis with phage α and phage βLC yielded a few particles of phage β.

Titrations of phage. Titrations of phage on non-lysogenic strains, and on strains lysogenic with phage βLC were, in general, made by the agar layer method (Hershey, Kalmanson & Bronfenbrenner, 1943). The agar layer method could not be used for titration of phage α on bacterial strains lysogenic with β because of the frequency with which these strains themselves produced phage α; with these strains, a modified Miles & Misra method was used (Miles & Misra, 1938), the areas of the plate which had not been inoculated with phage acting as a control for spontaneous plaques. In the modified Miles & Misra method, well-dried agar plates were sown for confluent lawns by flooding with suspensions of bacteria, the excess being pipetted off. When the surface had dried, 0·02 ml. drops of phage suspension were spotted on marked areas. With both methods, the plaques were counted after the plates had been incubated at 37° for 24 hr. The efficiency of plating of the phages on non-lysogenic strains was the same by the two methods of titration. Except where otherwise stated, the phage titres given are the concentrations of plaque-forming particles on non-lysogenic strain Davis.

For assaying free phage in the presence of lysogenic or infected bacteria, streptomycin-resistant variants of strain Davis or strain 2160S, or their derivatives lysogenic with βLC, were used as indicators, streptomycin being added to the agar medium to a concentration of $100\,\mu g$/ml. (Bertani, 1951). The efficiency of plating of free phage on the streptomycin-resistant variants on streptomycin agar was the same as on the streptomycin-sensitive strains on agar without streptomycin. Plaques were not produced on streptomycin-resistant indicators on streptomycin agar by infected streptomycin-sensitive bacteria early in the latent period, but it seemed possible that they might be produced not only by free phage, but also by some, or perhaps all, the infected bacteria late in the latent period, when the bacteria contained mature phage particles. The minimum latent period of phage γ in strain Davis was about 65–70 min. and control experiments in which infected bacteria were passed through anti-phage serum to neutralize any phage released early showed that 60 min. after infection about half the infected bacteria were able to produce plaques on streptomycin agar.

Anti-phage sera. These were produced by intravenous inoculation of rabbits with phage grown on strain Davis. The rabbits received weekly inoculations of 1 ml. of broth culture filtrates of titre about 5×10^9 particles/ml. for 6 weeks or more.

RESULTS

Temperate and virulent characters of the phages

Neither phage α nor phage γ established lysogenicity; secondary growth arising when sensitive strains were lysed by these phages was never found to be lysogenized by them. Resistant growth owed its resistance to failure to absorb the phage. Lysogenic bacteria producing phage β or phage βLC could be obtained when sensitive strains were exposed to phage β or phage βLC respectively. These absorbed the phage as readily as the parent non-lysogenic strains.

Phages α and γ formed clear plaques indistinguishable from each other on non-lysogenic strain Davis (Pl. 1, fig. 1). Although phage β formed clear plaques on strain Davis on ordinary nutrient agar, it formed plaques containing bacterial growth on agar supplemented with liver extract. The plaques of phage β on liver-extract agar were target-like in appearance, each having a clear centre surrounded by a ring of bacterial growth separated from the main lawn by a narrow line of clearing (Pl. 1, fig. 1). Of various other supplements tried (yeast extract, various amino acids, purines, nicotinamide, $MnCl_2$, $MgSO_4$, $FeSO_4$, $ZnSO_4$, $CoCl_2$, $CaCl_2$, KCl, Na citrate, Na oxalate) only manganese, known to influence the metabolism of *Bacillus* spp. (Weinberg, 1955), permitted the formation of target plaques by phage β; 2×10^{-6}M-$MnCl_2$ was sufficient with one batch of Lemco agar. Addition of manganese also promoted thicker growth of the lawns of strain Davis.

The plaques of phage βLC on strain Davis had a central area of thick growth surrounded by a fairly wide clear rim (Pl. 1, fig. 2).

Characters of bacterial strains artificially lysogenic with phage β

Lysogenic derivatives of seven bacterial strains were examined. These were the atypical *Bacillus anthracis* strains, Davis, Lindsay and 2160S; the typical *B. anthracis* strains, 2160, 1444 and H_3; and *B. cereus* 1651. All the lysogenic derivatives of these strains resembled the naturally lysogenic *B. cereus* strain W in that phage α, but not phage γ or phage β, formed plaques on them, and in that they regularly yielded phage β and sometimes phage γ and phage α. Phage α was found in broth cultures incubated for several days and, in addition, a few phage α plaques sometimes appeared when the bacterial strains were grown on solid medium. With the artificially lysogenic strains as well as with the naturally lysogenic strain W, these spontaneous plaques were much more frequent on nutrient agar supplemented with liver extract than on nutrient agar without this supplement. The phage was adsorbed rapidly to the atypical strains of *B. anthracis*, and slowly to the typical strains and to *B. cereus* strains 1651 and W. Spontaneous phage α plaques were much more frequently seen with the atypical strains of *B. anthracis* than with the others.

Strain Davis. Stable lysogenic bacteria of this strain could be obtained by subculture either of the ring of growth in the plaques of phage β or of the secondary growth in broth cultures cleared by phage β. The first subculture from either source was eroded, suggesting that the rate of stable lysogenization was low. Boyd (1951) found that with the group A phages of *Salmonella typhimurium*, a high phage:bacterium ratio increased the rate of lysogenization sufficiently to prevent clearing of broth cultures, but with phage β no such effect was seen.

The appearance of the growth of all of about 30 isolates of strain Davis (β) differed markedly from that of non-lysogenic strain Davis (Pl. 1, figs 3, 4). The confluent growth was thinner and sometimes showed vitreous patches of partial clearing; the isolated colonies were also thinner and usually had a central spot of partial clearing. These areas of partial clearing were not caused by phage α spontaneously appearing.

Strain Davis lysogenic with β contrasted markedly with strain Davis lysogenic with βLC. Phage βLC evidently established stable lysogenicity more readily than phage β, for the first subculture from plaques of phage βLC did not give eroded growth, and lysogenicity with βLC did not alter the cultural characteristics of strain Davis (Pl. 1, fig. 5). A further difference was observed when the production of phage in broth cultures of the two lysogenic derivatives was compared. At comparable bacterial densities, very much more phage was present in cultures of strain Davis (β) than in cultures of strain Davis (βLC), and the curves of phage production by the two strains were different; one experiment is illustrated in Fig. 1*a*, *b*. The concentration of free phage in cultures of either strain rose steadily until a certain bacterial density was reached, after which it fell, presumably because the rate at which phage was adsorbed to bacteria now became greater than the rate at which it was liberated. As cultures of strain Davis (βLC) continued to grow, the fall in free phage concentration continued, but with cultures of strain Davis (β), a second rise in phage concentration occurred. Since phage was evidently reabsorbed, the second rise with strain Davis (β) might have been the result of phage multiplication caused by superinfection of the lysogenic bacteria. Experiments were therefore made to test the effect of superinfection of lysogenic strain Davis with phage β. These experiments (described later) showed that superinfection of strain Davis (β) with phage β did in fact cause increased amounts of phage to be produced, and that phage β had some ability to induce its corresponding prophage.

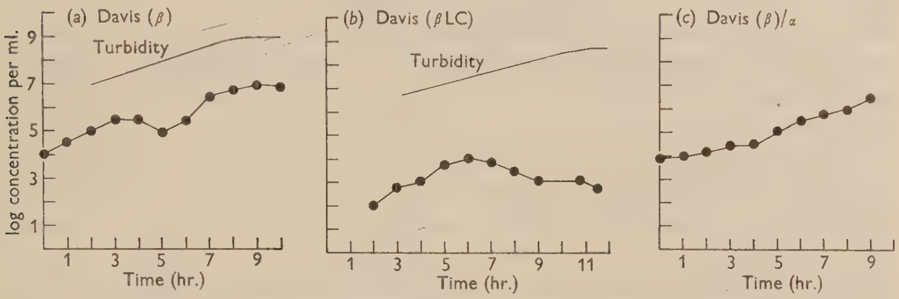

Fig. 1. Spontaneous production of phage in broth cultures of strains Davis (β), Davis (βLC) and a variant of Davis (β) not absorbing phage β. Cultures of strains Davis (β), Davis (βLC) and a variant of strain Davis (β), Davis (β)/α, not absorbing phage β, in 50 ml. volumes of broth in 250 ml. conical flasks were incubated at 37°. The inoculum in each case was 0·5 ml. of an 18 hr. broth culture. Phage production was followed by plating samples with streptomycin-resistant strain Davis on streptomycin agar. Bacterial growth of strains Davis (β) and Davis (βLC) was followed nephelometrically; the positions of the growth curves are only approximate and were estimated from comparisons with turbidities of cultures of *Escherichia coli* on which viable bacterial counts were made. The growth curve of the non-absorbing variant could not be measured either by nephelometry or colony counts, for it grew in long chains which formed a tangled mass at the bottom of the flask. As far as it could be judged by eye, the culture grew at about the same rate as the cultures of strains Davis (β) and Davis (βLC).

A variant of strain Davis (β) which did not absorb phage β was selected by exposing strain Davis (β) to phage α. The concentration of free phage increased steadily in cultures of this variant (Fig. 1*c*), supporting the idea that the second rise seen in cultures of phage-absorbing strain Davis (β) was due to induction caused by superinfection and not to spontaneous induction happening more often in the later stages of growth.

Alteration of the colonial appearance of a strain with lysogenicity has been reported by Ionesco (1953). Strain Davis (β) was evidently not completely immune to phage β. It thus seems possible that no further explanation is needed for the altered cultural appearance of strain Davis (β) (thinner growth and hollowed centres of the colonies) than the fact that this lysogenic strain can undergo what is virtually an autolysis by producing phage to which it is itself susceptible.

Strains other than strain Davis. All of 3 separate isolates of lysogenic *Bacillus anthracis* strain H_3, 5 separate isolates of lysogenic *B. anthracis* strain 1444 and 2 separate isolates of lysogenic *B. cereus* strain 1651, resembled their parent strains in colonial morphology and in forming spores. Four out of 5 separate isolates of lysogenic *B. anthracis* strain 2160 also resembled the parent strain in these characters; the fifth isolate, however, formed smoother colonies, was asporogenous and absorbed the phage rapidly, in contrast to the parent strain and its other lysogenic derivatives. This exceptional isolate of lysogenic strain 2160, which has been called 2160 (β) S, was picked because of its aberrant colonial appearance, from the secondary growth on a plate on which strain 2160 had been exposed to phage β. Since the other isolates of strain 2160 (β) resembled the parent strain, it seems unlikely that the altered characters of this isolate were due to its lysogenicity. Efforts to rid it of its lysogenicity failed, but it seems probable that it was a smooth variant of strain 2160 which had been lysogenized, for variants of non-lysogenic strain 2160 resembling it in colonial morphology, rapid adsorption of phage and failure to spore were obtained by incubating strain 2160 at 37° for 7 days in broth containing 0·025 %, w/v, $CaCl_2$ (Bordet & Renaux, 1930; Renaux, 1952). These variants (2160S) were not altered in their colonial characteristics by lysogenization. Non-lysogenic *B. anthracis* strain Lindsay formed smooth colonies and was asporogenous; lysogenization did not alter its colonial characteristics.

Phage β adsorbed rapidly to the atypical *Bacillus anthracis* strains Davis, 2160S and Lindsay, but exceedingly slowly to the typical *B. anthracis* strains H_3, 1444, and 2160 and to *B. cereus* 1651. Slow adsorption might have accounted for the failure of lysogenicity with phage β to alter the colonial appearance of these latter strains, but could not account for its failure to alter the appearance of strains 2160S and Lindsay. The growth of these two strains was more luxuriant than that of strain Davis, and so perhaps any effect on their colonial appearance caused by lysis through superinfection was counteracted by the more luxuriant bacterial growth.

Attempts to induce lysogenic strains with ultraviolet radiation and nitrogen mustard. Attempts were made to induce the naturally lysogenic strain W and the artificially lysogenic strain Davis (β) with ultraviolet radiation. It was clear that prophage β was inducible to some extent, for an increase of phage up to 40-fold of that in a control unirradiated culture was obtained on several occasions with strain W, and a small increase was also obtained with Davis (β). However, although a number of different conditions of cultivation were tried with strain W, and bacteria at different stages of their growth were tested, satisfactory induction was never obtained; attempts to induce strain W with nitrogen mustard failed.

Ability of phage α to attack lysogenic strains

Lysogenicity with phages β or βLC conferred little immunity against phage α; phage α formed plaques on the naturally lysogenic *Bacillus cereus* strain W and on the artificially lysogenic derivatives of sensitive *B. anthracis* and *B. cereus* strains, and its efficiency of plating on the lysogenic derivatives of strains Davis and 2160S was about half that on the non-lysogenic parent strains. In one-step growth experiments, phage α had the same minimum latent period in strain Davis (β) and strain Davis (βLC) as in non-lysogenic Davis, and showed the same curve of increase of plaque-forming particles. Fig. 2 shows the results of an experiment in which growth of phage α in strain Davis (βLC) with low multiplicity of infection was followed for 155 min. and compared with growth in non-lysogenic strain Davis up to 90 min. Samples from the infected cultures were plated with streptomycin-resistant strain Davis (βLC) on streptomycin agar as well as with strain Davis (βLC) on agar without streptomycin. As mentioned previously (Methods), not only free phage but also infected streptomycin-sensitive bacteria once they contain mature phage produce plaques on

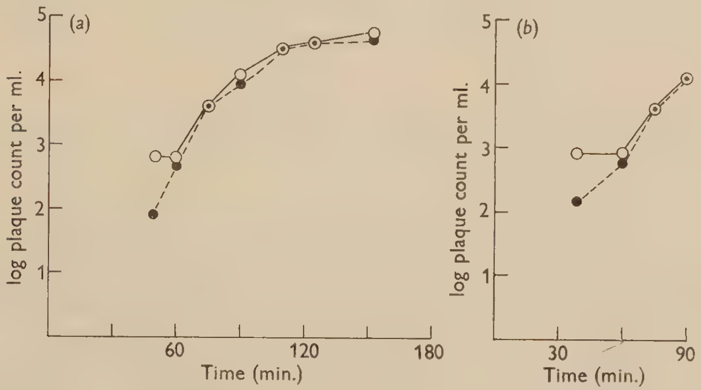

Fig. 2. Growth curves of phage α in strain Davis (βLC) and non-lysogenic strain Davis. Phage α at a concentration of 9×10^6 particles/ml. was mixed at 37° with broth suspensions of strain Davis (βLC) and non-lysogenic strain Davis of turbidity approximately corresponding to that of 5×10^8 *Escherichia coli*/ml. After 10 min. for adsorption, the mixtures were treated with anti-phage serum and equally diluted into broth at 37°. Samples taken at intervals were plated with strain Davis (βLC) on agar without streptomycin and with streptomycin-resistant strain Davis (βLC) on streptomycin agar. (a) = growth curve in strain Davis (βLC). (b) = growth curve in non-lysogenic strain Davis. ○ = plaques on agar without streptomycin. ● = plaques on streptomycin agar.

streptomycin agar. In the present experiment, the ratio of plaques on streptomycin agar to plaques on agar without streptomycin at 40 min. was the same with both hosts, and it may be inferred that the same proportion of infected bacteria of both strains contained mature phage by this time. These findings suggest that phage α started to multiply as soon in lysogenic as in non-lysogenic strain Davis.

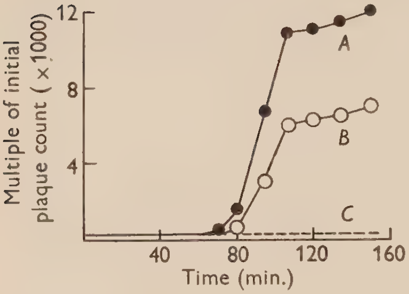

Fig. 3

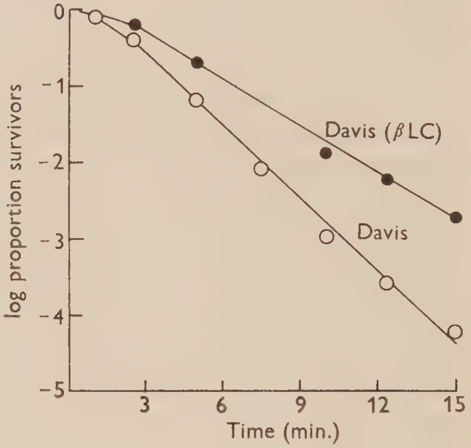

Fig. 4

Fig. 3. Growth curve of phage α in strain Davis (β). A suspension of strain Davis (β) was divided into two parts, phage α was added to one and, after 3 min. for its adsorption, both parts were treated with anti-phage serum to neutralize the free phage present. The multiplicity of infection with phage α was 9·8/chain of bacteria. Suitable dilutions of both suspensions in broth were incubated at 37°, and samples taken from them at intervals were plated with non-lysogenic strain Davis on agar without streptomycin. The plaque counts expressed as multiples of the initial number at 15 min. are plotted against time after the adsorption mixture was made. Curve *A* shows the numbers of phage α plaques and curve *B* the numbers of phage β plaques produced by the culture superinfected with phage α. Curve *C* shows the numbers of plaques (all β) produced by the control culture without phage α.

Fig. 4. Restoration of ultraviolet-inactivated phage α in strain Davis (βLC). A suspension of phage α in Ringer's solution was exposed to the radiation from a Hanovia 'Bactericidal' 15 W. lamp emitting 95 % of its radiation at 2537 Å. Samples taken at intervals were plated with non-lysogenic strain Davis and with strain Davis (βLC), the same concentration of plating bacteria being used in the two cases.

Fig. 3 shows the numbers of α plaques and β plaques produced after a culture of strain Davis (β) had been superinfected with phage α at a multiplicity of 9·8/chain, together with the numbers of β plaques produced by a control culture of strain Davis (β) not infected with phage α. At the beginning of the experiment, the number of plaques was equal to the calculated number of chains present, therefore, since the free phage had been neutralized by antiserum, it appeared that each chain of strain Davis (β) produced a plaque. Three sorts of plaque were seen with the culture infected with phage α, namely clear, target and mottled. A number of the mottled plaques were tested and found to give both phage α and phage β. Each mottled plaque was presumed to have arisen from an infected chain which liberated both sorts of phage, for at

15 min. the total number of plaques equalled the calculated number of chains, and until 60 min. almost all the plaques were mottled. After this time, when the number of plaques started to increase markedly, the mottled plaques formed a decreasing proportion of the total. The plaque counts have been expressed as multiples of the initial count at 15 min. and have been plotted against time elapsing since the phage and bacteria were mixed; the phage α counts represent the sum of clear and mottled plaques and the phage β counts the sum of target and mottled plaques. Infection by phage α evidently induced the prophage in strain Davis (β), for there was an increase of phage β in the culture infected with phage α which paralleled the increase of phage α and which greatly exceeded its increase in the control culture not infected with phage α. A similar result was obtained when the naturally lysogenic strain W was infected with phage α.

Ultraviolet-irradiated phage α produced more plaques on lysogenic strain Davis than on non-lysogenic strain Davis (Fig. 4), whereas unirradiated phage α produced only about half as many plaques on lysogenic strain Davis as on non-lysogenic strain Davis.

Jacob & Wollman (1953) concluded that the reason why the virulent mutant $\lambda\nu$ of the coliphage λ can form plaques on strains lysogenic with temperate λ is because it can induce the prophage to develop into phage, and in this way convert immune bacteria into sensitive ones in which it can multiply together with the temperate phage. The bacillus phage α resembled coliphage $\lambda\nu$ both in having inducing ability and in being restored by lysogenic strains after inactivation by ultraviolet radiation. It is therefore likely that both phages owe their ability to attack lysogenic hosts to the same cause, and that the arguments applied to coliphage $\lambda\nu$ (Jacob & Wollman, 1953) apply equally to bacillus phage α: that is, that the virulent phage induces the prophage before it multiplies, and that it is as a consequence of this inducing ability that it is able to multiply in lysogenic strains.

Inducing activity of phage γ

Phage γ did not form plaques on strains lysogenic with β or βLC. However, drops of undiluted stocks of phage γ spotted on lysogenic strain Davis or lysogenic strain 2160S produced patches of complete clearing, and the clearing became less complete with dilution, the effect resembling that obtained with serial dilutions of a colicine. This activity of phage γ suspensions was proportional to the concentration of particles forming plaques on non-lysogenic strain Davis, and was destroyed by addition of anti-phage serum. On centrifuging phage preparations at high speed, the activity travelled with the plaque-forming particles (Table 1). Superinfection of strain Davis (βLC) with phage γ increased the total phage production, the additional phage consisting of both γ and βLC. In increasing the total phage production, superinfection with phage γ differed from superinfection in systems where immunity is evidently complete (Bertani, 1953, 1954; Jacob & Wollman, 1953), and resembled superinfection with phage α. Fig. 5 shows the results of an experiment in which the rate of phage production in a culture of strain Davis (βLC) superinfected with

Table 1. *Effect of concentrated phage γ on strain Davis (βLC)*

Ten ml. of a stock of phage γ were centrifuged in a Spinco Ultracentrifuge at 21,400 *g*. for 90 min. The uncentrifuged stock, the top 1 ml. of the supernatant fluid, and the deposit resuspended in 1 ml. of broth were assayed with strain Davis, and 0·02 ml. drops of serial tenfold dilutions were spotted on plates inoculated with strain Davis (βLC)

	Dilution	Calculated number of plaque-producing particles/drop	Effect on strain Davis (βLC)
Uncentrifuged stock (titre on strain Davis $=1\times10^{10}$ particles/ml.)	10^0	2×10^8	Complete clearing
	10^{-1}	2×10^7	Complete clearing
	10^{-2}	2×10^6	Partial clearing
	10^{-3}	2×10^5	Trace
	10^{-4}	2×10^4	No mark
Supernatant fluid (titre on strain Davis $=5\times10^8$ particles/ml.)	10^0	1×10^7	Complete clearing
	10^{-1}	1×10^6	Trace
	10^{-2}	1×10^5	No mark
Deposit (titre on strain Davis $=5\times10^{10}$ particles/ml.)	10^0	1×10^9	Complete clearing
	10^{-1}	1×10^8	Complete clearing
	10^{-2}	1×10^7	Complete clearing
	10^{-3}	1×10^6	Trace
	10^{-4}	1×10^5	No mark

phage γ at a multiplicity of 0·1/chain was compared with that in a control culture without phage γ. Samples were plated with streptomycin-resistant strain Davis on streptomycin agar to eliminate the background of plaques produced by chains of lysogenic bacteria. It will be seen that phage γ is able to some extent to induce the development of prophage βLC to phage, for more phage βLC was produced in the culture superinfected with phage γ, and that because of this inducing ability it is itself able to multiply to some extent, for the γ plaques increased in parallel with the extra βLC plaques.

The growth curve of phage γ in strain Davis (βLC) differed considerably from that of the phages in systems where plaques were formed, e.g. any of the phages in non-lysogenic strain Davis or phage α in lysogenic strain Davis. In one-step growth experiments with any of the phages in non-lysogenic strain Davis or phage α in lysogenic strain Davis, the minimum latent period was about 65 min.* Three striking differences could be observed between the growth curve of phage γ in strain Davis (βLC) and the growth curves of the phages in systems where plaques were formed. (1) Only a small proportion (1/350) of the bacteria which had adsorbed phage γ gave rise to plaques on streptomycin agar at 60 min. suggesting that only about this proportion then contained mature phage (see Methods). With systems where plaques were formed, half or more of the infected bacteria would have produced plaques on streptomycin agar by this time. This suggests that not more than 1/175 of the bacteria which had absorbed phage γ contained mature phage by this time. (2) Even as late as 255 min. the increase of phage over that initially absorbed was only 12-fold, whereas with systems where plaques were formed it was about 100-fold at 120 min. (3) With systems where plaques were formed, a decline in the rate of increase of plaque-forming particles occurred at 100–120 min.; in this case, the number of plaques was still increasing linearly at 210 min. It seems very unlikely that the phage γ present at 210 min. was derived solely from those bacteria which contained mature phage γ at 60 min.; more probably intracellular maturation of phage γ in strain Davis (βLC) was not synchronized to the same extent as that of phage in systems where plaques were formed.

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* The technique of the one-step growth experiment does not give true one-step growth curves when the host grows in chains, for phage particles released in the first burst re-adsorb to other bacteria in the chain from which they came, and go through a further cycle of multiplication, and this second cycle of multiplication cannot be avoided by dilution as in the ordinary one-step growth curve (McCloy, to be published.) However, in one-step growth experiments with any of the phages in non-lysogenic strain Davis or phage α in lysogenic strain Davis, a marked decline in the rate of increase of plaque-forming particles occurred at about 100 min., probably indicating that the majority of the bacteria infected in the first cycle had released their phage by this time; and from the number of plaques at, or soon after 100 min., the mean burst size could be estimated to be of the order of 100.

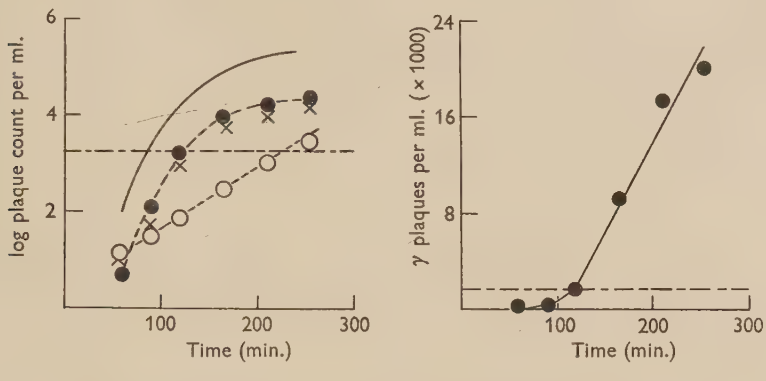

Fig. 5*a*, *b*. Superinfection of strain Davis (βLC) with phage γ. A broth culture of strain Davis (βLC) in the logarithmic phase of growth was centrifuged, the deposit was re-suspended in a smaller volume of fresh broth to a concentration of $1{\cdot}25 \times 10^8$ chains/ml. as estimated by colony count, and divided into two 0·9 ml. volumes. 0·1 ml. of phage γ was added to one giving a concentration of $1{\cdot}75 \times 10^7$/ml., and 0·1 ml. of broth was added to the other. After 10 min. for adsorption, both suspensions were treated with anti-phage serum to inactivate the free phage present. 99 % of the phage γ was absorbed, as estimated by centrifuging a dilution made without anti-phage serum and assaying the supernatant. Suitable dilutions of each suspension in broth were incubated at 37°, and samples taken at intervals were plated with streptomycin-resistant strain Davis on streptomycin agar.

(*a*) shows log concentration of γ plaques (●) and βLC plaques (×) produced by the superinfected culture, together with βLC plaques produced by the control culture (○) plotted against time after addition of phage γ.

(*b*) shows the arithmetic concentration of γ plaques (●) plotted against time after addition of phage γ.

Continuous lines. Theoretical curves for the increase of phage γ in cultures of strain Davis (βLC) derived on the assumptions (1) that the phage in 1/175 of the infected bacteria reaches maturity every 5 min. from 60 min. onwards, and (2) that, after maturation, infective centres increase at the same rate as in systems where plaques are formed. The curve in (*a*) has been displaced upwards by one log unit for clarity.

Broken lines. Concentration of phage γ adsorbed to the bacteria.

It is clearly impossible from a curve such as that of Fig. 5 to determine what proportion of the infected bacteria did eventually burst, but to get some idea of what proportion of bacteria of strain Davis (βLC) which had absorbed phage γ did ultimately release phage γ, suspensions of bacteria of strain Davis (βLC) were infected with phage γ at low multiplicity, and, after dilution through anti-phage serum, plated with non-lysogenic strain Davis on agar without streptomycin. After incubation, a number of normal-sized clear plaques and a few normal-sized mottled plaques could be distinguished and counted fairly satisfactorily, despite the presence of a background of small plaques arising from micro-colonies of the lysogenic strain. Control suspensions of strain Davis (βLC) without phage γ, plated at the same time, produced neither clear nor mottled plaques. In one experiment, $1{\cdot}4 \times 10^4$ γ particles were absorbed and $2{\cdot}8 \times 10^3$ normal-sized clear plaques resulted; in another experiment $9{\cdot}8 \times 10^4$ γ particles were absorbed and $1{\cdot}6 \times 10^4$ normal-sized clear plaques resulted. Thus, in the first experiment 21 %, and in the second experiment 16 % of the γ particles which adsorbed to bacteria of strain Davis (βLC) multiplied soon enough to give normal-sized plaques when plated with non-lysogenic strain Davis.

The small increase in phage γ over the input in the experiment of Fig. 5 might be the result of the bursting of all of this fraction during the period of the experiment (255 min.), with a smaller burst size and a latent period which was more variable, but only slightly longer than the usual. Or it might be the result of a situation where the burst size was of the usual order, and where only a proportion of the bursts occurred within the period of observation, the period between infection and bursting varying between the normal and at least 255 min., at which time the plaque count was still rising. From 120 to 210 min. the plaque count increased approximately linearly with time (the curvature of the line in Fig. 5*a* results from the use of semi-log co-ordinates as may be seen by comparison with Fig. 5*b* which shows the same data plotted arithmetically). Thus phage was being released at a constant rate instead of all at about the same time as in a normal one-step growth curve. It is possible that superinfecting phage γ becomes mature in a constant proportion of the infected bacteria/unit time, and that once maturation has started the cell bursts after the same interval and with the same burst size as in systems where plaques are formed; the curve of increase of plaques predicted by a model of this sort, assuming that the phage in 1/175 of the infected bacteria reaches maturity every 5 min. from 60 min. onwards, is shown as the continuous lines in Fig. 5*a* and 5*b*, and is very similar to the curve actually observed.

Since the latent period of phage α is the same in strain Davis (βLC) as in non-lysogenic strain Davis (Fig. 2), one may assume that the induction process initiated by phage α occupies a negligible time, and that its latent period of actual multiplication is no longer in strain Davis (βLC) than in non-lysogenic strain Davis. It is reasonable to suppose that the same is true of phage γ, and hence that the slow rate of intracellular maturation of phage γ is due to delay in causing induction after entry into the bacterium. Perhaps not all the particles which eventually induce start to induce at once. Particles of phage γ when they invade bacteria carrying prophage may remain viable, like other superinfecting phages, not inert as in systems where immunity is complete (Bertani, 1953, 1954; Jacob & Wollman, 1953) but with a certain probability/unit time of inducing the prophage to develop into phage and subsequently multiplying. The slow rate of growth of phage γ in strain Davis (βLC) is probably responsible for its failure to form plaques on this strain. This hindrance might be overcome if a large number of phage particles were inoculated at one point. This was done by plating bacteria of non-lysogenic strain Davis infected with phage γ, each of which would liberate *c.* 100 phage particles at one point, instead of free phage γ, and visible though very small plaques were in fact produced (Pl. 1, fig. 6). Their numbers in two experiments were 25 and 50% of the numbers of plaques produced on non-lysogenic strain Davis.

Inducing activity of phage β

Although phage β did not form plaques on bacterial strains lysogenic with β itself or with βLC, drops of concentrated phage β spotted on lysogenic strains Davis or 2160S produced patches of clearing resembling those produced by phage γ, the activity of suspensions being proportional to their concentration of plaque-forming particles on non-lysogenic strain Davis. As can be seen in Table 2, the activity of phage β per plaque-forming particle was about 10 times less than that of phage γ. The clearing was due to phage β itself and not to the presence of phage γ contaminating the suspensions.

Table 2. *Effect of concentrated phage β on strain Davis (βLC)*

Two 10 ml. volumes of phage β were centrifuged in a Spinco Ultracentrifuge at 21,400 *g.* for 90 min. The uncentrifuged stock, the pooled supernatant fluids, and the pooled deposits resuspended in 1 ml. of broth, were assayed with strain Davis, and 0·02 ml. drops of serial 10-fold dilutions were spotted on plates spread with strain Davis (βLC).

	Dilution	Calculated number of plaque-producing particles/drop	Effect on strain Davis (βLC)
Uncentrifuged stock (titre on strain Davis $=1{\cdot}1\times10^{10}$ particles/ml.)	10^0	2×10^8	Partial clearing
	10^{-1}	2×10^7	Partial clearing
	10^{-2}	2×10^6	Trace
	10^{-3}	2×10^5	No mark
Supernatant fluid (titre on strain Davis $=4{\cdot}3\times10^8$ particles/ml.)	10^0	$8{\cdot}6\times10^6$	Trace
	10^{-1}	$8{\cdot}6\times10^5$	No mark
Deposit (titre on strain Davis $=1{\cdot}8\times10^{11}$ particles/ml.)	10^0	$3{\cdot}6\times10^9$	Complete clearing*
	10^{-1}	$3{\cdot}6\times10^8$	Complete clearing*
	10^{-2}	$3{\cdot}6\times10^7$	Partial clearing
	10^{-3}	$3{\cdot}6\times10^6$	Trace
	10^{-4}	$3{\cdot}6\times10^5$	No mark

* Or with a few colonies on a clear base.

The ability of phage γ to induce and to multiply in strain Davis (βLC) suggested that the clearing produced by phage β was due to the same cause. This hypothesis was supported by the shape of the curve of phage production in cultures of strain Davis (β) (Fig. 1*a*). In such cultures, after a period during which the titre of phage β decreased, evidently as a result of re-adsorption to the bacteria, there was a second rise in the concentration of phage, presumably due to induction following superinfection. Furthermore, it was shown that superinfection of strain Davis (β) by added phage β increased the production of phage β (Fig. 6). This effect might conceivably be due to the added phage β infecting bacteria which had spontaneously become non-lysogenic (Clarke, 1952; Bertani, 1958), but since the spontaneous rate of production of phage β by strain Davis (β) and the rate of adsorption of phage β to strain Davis (β)

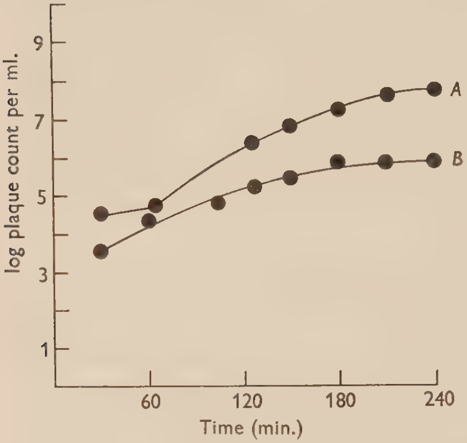

Fig. 6

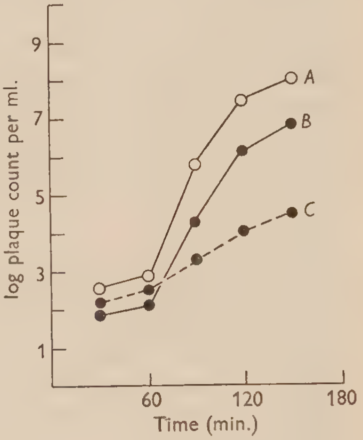

Fig. 7

Fig. 6. Superinfection of strain Davis (β) with phage β. A broth culture of strain Davis (β) towards the end of the logarithmic phase of growth was centrifuged, and the deposit was resuspended in fresh broth to a turbidity approximately corresponding to that of 5×10^8 *Escherichia coli*/ml. One ml. was placed in each of two tubes, and 0·05 ml. of a stock of phage β was added to one giving a concentration of $8{\cdot}5 \times 10^9$/ml. After 8 min. for adsorption of the added phage, both suspensions were treated with anti-phage serum to inactivate the free phage present. 1/200 dilutions of the suspensions in 50 ml. volumes of broth were incubated at 37°, and samples taken at intervals were diluted and plated with streptomycin-resistant strain Davis on streptomycin agar.

The log concentrations of plaques (all β) produced by the superinfected culture (curve *A*), and by the control culture (curve *B*) are plotted against time after addition of phage β.

Fig. 7. Superinfection of strain Davis (βLC) with phage β. A broth culture of strain Davis (βLC) in the logarithmic phase of growth was centrifuged, and the deposit was resuspended in fresh broth to a turbidity approximately corresponding to that of 10^9 *Escherichia coli*/ml. Equal volumes were placed in each of two tubes, and phage β was added to one to a concentration of 8×10^9/ml. After 10 min. for adsorption of the added phage, both suspensions were treated with anti-phage serum to inactivate the free phage present. Suitable dilutions in broth were incubated at 37°, and samples taken at intervals were plated with streptomycin-resistant strain Davis on streptomycin agar. The log concentrations of β plaques (curve *A*), and βLC plaques (curve *B*) produced by the superinfected culture, together with βLC plaques produced by the control culture (curve *C*) are plotted against time after addition of phage β. (A number of minute plaques were observed with both cultures in this experiment, and in other experiments where lysogenic strains were plated with streptomycin-resistant strain Davis on streptomycin agar. These have not been included in the counts given. Their significance is uncertain, but there is some evidence which suggests that they may be due to the occurrence of reversible adsorption of phage to lysogenic strains.)

are both high, it is unlikely that non-lysogenic bacteria, if they arose, would remain uninfected by phage β naturally present in the culture. It follows that the increase in phage β was probably a sequel of superinfection of lysogenic bacteria and induction. This was proved by superinfecting strain Davis (βLC) with phage β, for it was then found that both phages contributed to the excess of phage found by comparison with a control culture of strain Davis (βLC) without phage β (Fig. 7).

Appearance of the phages in cultures of lysogenic strains

Phage β

Phage β was produced regularly during growth of the naturally lysogenic strain W and of artificially lysogenic strains.

Phage γ

Phage γ could be detected during a certain period of the growth of broth cultures of bacterial strains lysogenic with phage β started from inocula in which the free phage had been inactivated, either by using a heated spore suspension, or, in the case of the asporogenous strains, by treating them with anti-phage serum. When samples from such cultures were plated with non-lysogenic streptomycin-resistant indicator strains, Davis or 2160S, on streptomycin agar, there were some clear plaques as well as the usual target-like β plaques. A number of these clear plaques were tested; all gave phage which formed clear plaques on non-lysogenic strain Davis, and did not form plaques on either lysogenic strain Davis or strain W, and which was thus by definition phage γ. Clear plaques caused by phage γ were only rarely seen with cultures started from inocula in which the free phage β had not been inactivated, and which therefore contained more phage β in the initial stages of their growth.

When growing cultures of strain W started from inocula of heated spores were examined, it was seen that with the first samples to produce any plaques, the number of γ (clear) plaques was about a tenth to a twentieth the number of β plaques. As the cultures grew and the plaques became more numerous, the γ plaques formed a progressively diminishing proportion of the total, and soon after the cultures had gone out of the logarithmic phase of growth clear plaques could no longer be detected; there is no selective system for phage γ in the presence of a great excess of phage β. In experiments where the free phage in the inoculum was not inactivated, clear plaques were only occasionally seen. It thus appeared that phage γ could arise in lysogenic cultures, and that if there was no free phage present initially, it formed a considerable, but progressively diminishing, proportion of the total phage during the earlier phase of growth of the culture.

The appearance of phage γ in these cultures of strain W was presumably due to a mutation of phage β. If only phage β in the form of lytic phage could mutate, then it would be expected that the number of γ plaques would form a constant proportion of the number of β plaques instead of a diminishing proportion as was found. Ultraviolet irradiation could induce the naturally lysogenic strain W to some extent; when cultures at a stage in their growth when phage γ was detectable were irradiated, an increased production of phage β was obtained, sometimes up to 40-fold of that in a control unirradiated culture, but this rise in phage β was never accompanied by a rise in phage γ. The findings are most easily explained by assuming that mutation from β to γ occurs in the prophage, and that the γ thus produced multiplies as lytic phage, which is likely since phage γ cannot lysogenize. There is no selective system for phage γ in the presence of phage β, but a streptomycin-resistant indicator on streptomycin agar is a selective indicator for phage γ in the presence of prophage β. Assuming that mutation to γ occurred in both prophage β and phage β at a low rate, the mutation could be detected when there was enough prophage β to give some γ mutants but not enough phage β to mask them. The conditions in cultures starting from phage-free inocula would thus favour the detection of such a mutation.

This hypothesis was supported by the results of a single-burst experiment in which broth inoculated with heated spores of strain W was distributed in small volumes which, after a period of incubation, were plated with streptomycin-resistant indicator strain 2160S on streptomycin agar. If phage γ arose by mutation of prophage β and subsequently multiplied, the bacterium in which this occurred might yield only phage γ. If it arose only by mutation of lytic phage β, the bacterium in which the mutation occurred would be expected to yield both phages. In this experiment, 204 samples were plated and of these 109 gave no plaques, 91 gave only β plaques, 3 gave only γ plaques, and 1 gave both γ and β plaques. The proportion giving both γ and β plaques was no higher than that to be expected if the two events were independent of each other, which suggests that this tube contained the yield of more than one bacterium.

Phage α

In cultures of the naturally lysogenic strain W. Broth cultures of strain W incubated for prolonged periods regularly came to contain large amounts of phage α. Loopfuls plated on nutrient agar gave rise to bacterial growth with eroded patches from which phage α could be isolated, and phage α was also found in filtrates of the broth cultures, reaching concentrations of about 10^9 particles/ml. The time of incubation before a broth culture gave eroded growth on plating varied between 2 and 25 days, and was perhaps shorter when the volume of the culture was large. The method of plating a loopful does not show when phage α first makes its appearance in the culture but reveals it only when it has multiplied to the concentration necessary for particles to be present in the volume held by the loop, that is, to above about 10^3 particles/ml. Tests for the first appearance of phage α consisting of plating the whole of cultures of small volume could not satisfactorily be made because: (*a*) the appearance of a few plaques in a lawn of a strain W does not necessarily mean that the particles originating the plaques were present in the inoculum (see below); (*b*) phage α-sensitive strains other than strain W were also sensitive to a bacteriocine which strain W produces, and thus a strain lysogenic with βLC could not be used as an indicator for phage α with undiluted fully grown cultures of strain W.

Addition of glucose, mercaptoacetate, calcium, citrate or oxalate to Lemco broth did not seem to alter the time of appearance of phage α; but addition of manganese hastened its appearance (Table 3). Addition of manganese to the broth gave an increased final crop of bacteria as judged by turbidity measurements, but a still higher concentration of bacteria obtained by aeration did not hasten the appearance of detectable amounts of phage α. Nor did concentration of the bacteria by centrifuging 24 hr. cultures and incubating the deposits resuspended in smaller volumes (1/5 to 1/2) of the original medium. The concentrations of phage β in cultures with and without added manganese, at times before phage α was present in detectable amount, were not appreciably different. The effect of added manganese might have been on the rate of multiplication of phage α to a detectable concentration; but in experiments to test this addition of manganese did not noticeably increase the rate of multiplication of phage α added to log phase cultures or 24 hr. cultures of strain W.

Table 3. *Effect of volume and composition of medium on time of appearance of phage α in cultures of strain W*

	Number of cultures first showing phage α on (days of incubation at 37°):																				Total number of cultures tested
Day ...	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
100 ml. Lemco broth	.	3	3	6	6	11	5	.	1	1	.	.	.	.	.	.	.	1	.	2	39
100 ml. Lemco broth shaken	.	.	.	2	.	1	1	1	.	2	1	.	.	.	.	.	.	.	.	.	8
100 ml. Lemco broth + $MnCl_2$	1	9	5	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	16
5 ml. Lemco broth	.	.	1	1	.	1	3	3	1	.	3	.	.	.	4	.	.	.	.	1	18
5 ml. Lemco broth + $MnCl_2$	1	5	4	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	12

Spontaneous plaques of phage α were sometimes seen when inocula of strain W apparently containing no phage α were plated. These plaques were more numerous on nutrient agar containing liver extract than on unsupplemented nutrient agar, and were more numerous after incubation for 48 hr. than for 24 hr. Since bacterial growth was more profuse on liver agar than on ordinary nutrient agar, the more numerous plaques on liver agar did not necessarily mean a greater rate of production of phage α on this medium.

In cultures of the artificially lysogenic derivatives of sensitive bacterial strains. Broth cultures of the lysogenic derivatives of sensitive bacterial strains obtained by exposure to phage β also came to contain high concentrations of phage α when incubated for long enough. The time at which phage α was detectable by plating a loopful of the culture was earlier with the rapidly absorbing atypical *Bacillus anthracis* strains Davis (β), Lindsay (β) and smooth lysogenic derivatives of strain 2160 than with typical *B. anthracis* strains or *B. cereus* strains, phage α being not uncommonly present in large amount in cultures of the atypical *B. anthracis* strains after only 24 hr. of incubation. Spontaneous plaques caused by phage α were also seen in cultures on agar; they were especially frequent with the atypical *B. anthracis* strains.

Interconversions

Phage β to phage γ

As described above, phage γ could be found in cultures of bacterial strains lysogenic with phage β, apparently as a result of mutation in prophage β. Spontaneous mutation of lytic phage β to γ could not be demonstrated; while, as argued above, plating on streptomycin agar is a selective system for phage γ in the presence of an excess of prophage β, there is no selective system for phage γ in the presence of a great excess of phage β, so small amounts of phage γ could not be detected in stocks of phage β. A phage with the properties of phage γ arose from phage β when it was irradiated with u.v. radiation and plated with bacteria which had also been irradiated (Weigle, 1953). When phage β irradiated with a dose of ultraviolet radiation which gave a survival of 10^{-6} on plating with unirradiated strain Davis was plated with irradiated bacteria, the survival was 10^{-5}, and some of the plaques which were produced were clear. The clear plaques yielded phage which formed clear plaques on non-lysogenic strain Davis and no plaques on lysogenic strain Davis, and thus had the properties of phage γ.

Phage β to phage α

Phage α could be found in cultures of bacterial strains lysogenic with phage β. It could also be found in stocks of phage β grown on sensitive strains by plating with the selective indicator strains Davis (βLC) and 2160(β)S, though here it was only detected in small amounts, e.g. a proportion of about 1 in 2×10^6. There was probably more phage α present than was detected, for tests on the sensitivity of the assay system made by making parallel titrations of a stock of phage α diluted in broth and diluted in a stock of phage β of titre 5×10^8 particles/ml. showed that the presence of this concentration of phage β lowered the efficiency of plating of phage α to about 1/6 to 1/10, and caused the plaques to be small and indistinct. In contrast to the situation in cultures of lysogenic strains, phage α was evidently at no selective advantage over phage β in cultures of non-lysogenic bacterial strains. When a mixture of phage α and phage β was serially passaged five times with non-lysogenic strain Davis, with mass transfers of phage at each step, the relative proportions of the two phages remained the same throughout.

Phage α to phage β

Phage β was obtained from phage α only in one experimental system. Bacilli of sensitive strains could sporulate despite infection with phages α or γ (McCloy, 1953; and to be published). A large proportion, about $0{\cdot}5 - 0{\cdot}1$, of the spores of *Bacillus anthracis* strain 1444 formed in the presence of phage α were found to be lysogenic with phage β. Phage β could evidently arise during growth of phage α in strain 1444, for spores lysogenic with phage β were found when single plaques of phage α on strain 1444 were picked and replated with strain 1444.

No phage β was found when phage α was grown on strain Davis. The secondary bacterial growth in about 20 cultures of strain Davis which had been lysed by phage α was examined, but no bacteria lysogenic with phage β were found. In one experiment, 100 ml. of broth were inoculated with strain Davis and phage α, and incubated until lysis had been followed by secondary growth. The culture was then centrifuged, and half the supernatant fluid + an equal volume of fresh broth was inoculated with a large amount of strain Davis, and again incubated until the culture had cleared and secondary growth had appeared. Fourteen such subcultures were made and at each step the deposit was examined for bacteria lysogenic with phage β, but none were found.

Since strains lysogenic with phage β are still susceptible to phage α, the difference between the results in the two systems tested need not have been due to a difference in the frequency with which phage β arose from phage α in the two hosts; it might merely have been due to the difficulty of detecting phage β in the presence of phage α. Bacilli of *Bacillus anthracis* strain 1444 absorb the phage slowly, and so single infections might be less rare with this strain than with strain Davis which absorbs rapidly. Furthermore, a bacillus just about to sporulate might absorb only one phage particle before its sporulation was completed, and spores (which do not absorb) containing phage β would be completely protected from particles of phage α in their environment.

Phage α to phage γ

Since there was no selective indicator for phage γ in the presence of phage α, there were no means of detecting small amounts of phage γ in the presence of large amounts of phage α. There was not enough phage γ in stocks of phage α to be detected without a selective indicator; some hundreds of plaques produced when preparations of phage α were plated with non-lysogenic bacterial strains were examined, but none were found to contain phage γ.

Phage γ to phage β

When the secondary growth in each of about 30 broth cultures of strain Davis which had been cleared by phage γ was examined, bacteria lysogenic with phage β were found in two of them. Thus phage β could arise from phage γ though its appearance was only rarely observed. When the spores of *Bacillus anthracis* strain 1444 formed in the presence of phage γ were examined, no spores lysogenic with phage β were found, although an extensive search was made for them, whence it may perhaps be inferred that phage β arose from phage γ much more rarely than from phage α.

Phage γ to phage α

Stocks of phage γ could be tested for the presence of phage α by plating with indicator strains lysogenic with phages β or βLC, but only a limited concentration of phage γ could be plated because of the clearing which phage γ itself produced in high concentration. Phage α was found in many stocks of phage γ in a proportion of about 1 in 5×10^6.

Phage α was not observed to give rise to phage γ, but there was no selective indicator available to detect small amounts of phage γ in the presence of large amounts of phage α. With this one exception, each phage variant was observed to give rise to each of the other two without large amounts of the third being present. It may be inferred from this that each form of the phage could be obtained from either of the other two without passing through the third, which suggests that the determinants of the three forms were probably allelic.

DISCUSSION

Bacillus cereus strain W carries a temperate phage Wβ which, apparently as a result of a single mutation, gives rise to a variant Wα which can form plaques on strain W itself and on other bacterial strains lysogenic with the temperate phage. It can do this probably because it is able to induce the prophage to develop into phage, and thus overcome the immunity which is conferred on the bacteria by the prophage. Bacterial strains which give eroded growth are not uncommonly seen, and the isolation of such strains has been reported by several authors including Twort (1915), Scholtens (1950) and Fisk (1942). It is probable that these are lysogenic strains analogous to strain W in that the temperate phage they carry frequently mutates to an inducing virulent form. Evidently the lysogenic culture provides an enrichment medium for mutants of this sort. This mechanism might therefore explain the early reports of spontaneous generation of phage during serial passage of bacteria with supernatant fluids or filtrates from cultures of the same strain, and may in some cases have been misinterpreted as the adaptation by serial passage of an added phage to attack a given strain of bacteria.

It is conceivable that the phage carried by any lysogenic strain could mutate to an inducing virulent variant, the reason why such variants have been found with only a few phages being that the others are many mutational steps from the inducing virulent form. The genetics of phage λ have been fully analysed (Jacob & Wollman, 1954; Kaiser, 1955) and wild-type λ has been found to differ by several genes from the inducing virulent λv.

The genetic determination of characters in phage W evidently differs somewhat from that in phage λ; in phage λ there are three alleles at one locus, only one of which, $v1$, determines the ability to induce. Phages with either of the other two, c (determining clear plaques and inability to lysogenise) or c^+ (determining turbid plaques and ability to lysogenize), do not induce. The degree of inducing ability of phage with the gene v_1 (i.e. the probability per phage particle of inducing) is determined by genes at three other loci. In phage W, there are evidently three allelic genes β, γ and α, at one locus which appear superficially to correspond to c^+, c and v_1 in phage λ, from which however they differ in that each is associated with some inducing ability, the extent of this being determined by which is present. Even the β allele which lysogenizes is associated with some degree of inducing activity. The inducing activity of phage β in lysogenic cultures manifests itself by a form of autolysis when the bacterial strain absorbs the phage readily and is slowly growing. For example, the growth of strain Davis (β) is thinner than that of non-lysogenic strain Davis, and the centres of individual colonies are hollowed out. Although the effect of lysogenicity on the colonial morphology of the naturally lysogenic *Bacillus cereus* strain W could not be directly determined because no non-lysogenic derivatives of strain W were obtained, it seemed unlikely that carriage of phage β was affecting the colonial appearance of this strain since it resembled that of other strains of *B. cereus*. Lawns of strain W were occasionally seen which were finely pitted, and these pits may possibly have been associated with the inducing effect of phage β, for they were evidently not due either to spontaneously-appearing phage α or to sporulation.

With a few exceptions (Burnet & Lush, 1936; Bradley & Boyd, 1952; Boyd, 1954), the bacteria of lysogenic strains absorb the phage that they produce. However, in all systems reported so far, the lysogenic strain is immune to the phage that it produces (Bertani, 1958) although in most cases the only test for immunity that has been used is the ability of the phage to produce clearing of the lysogenic strain. In some cases where more detailed examination has been made (Bertani, 1954; Jacob & Wollman, 1953), it has been shown that superinfection with the phage does not result in any detectable increase in the rate of phage production by the lysogenic culture. Phage β provides a striking exception to the rule of immunity, for superinfection of strain Davis (β) with phage β causes a considerable increase in phage production. Generally, too, a lysogenic strain does not differ from its non-lysogenic relative in colonial appearance. The colonial appearance of strain Davis, which absorbs phage β rapidly, was grossly altered by lysogenicity with phage β, presumably because of its incomplete immunity. Dr Six (personal communication) has similarly found that in the case of the *Shigella shigae* phage *P* 2, the immunity of a lysogenic strain is not absolutely complete, for about one organism in 10^{-3} superinfected will lyse as a consequence of superinfection.

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EXPLANATION OF PLATE

Fig. 1. Plaques of phage α and phage β on strain Davis.
Fig. 2. Plaques of phage βLC on strain Davis.
Fig. 3. Colonies of non-lysogenic Davis.
Fig. 4. Colonies of Davis (β).
Fig. 5. Colonies of Davis (βLC).
Fig. 6. Plaques produced by γ-infected bacteria of non-lysogenic Davis on Davis (βLC).

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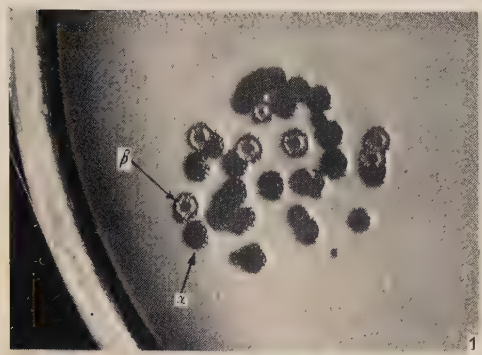

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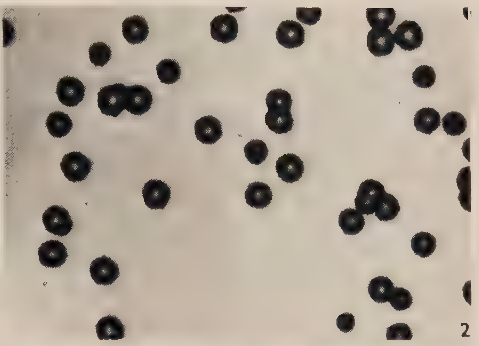

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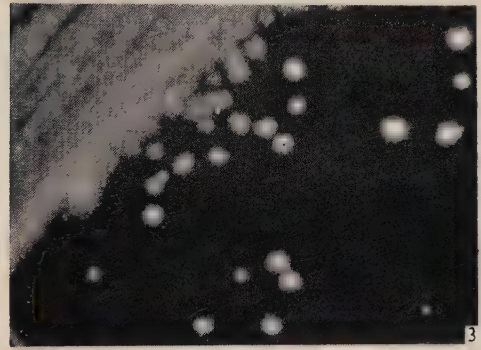

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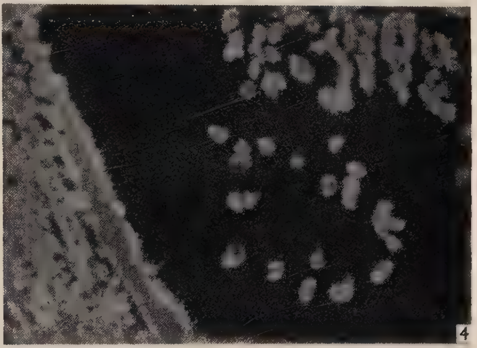

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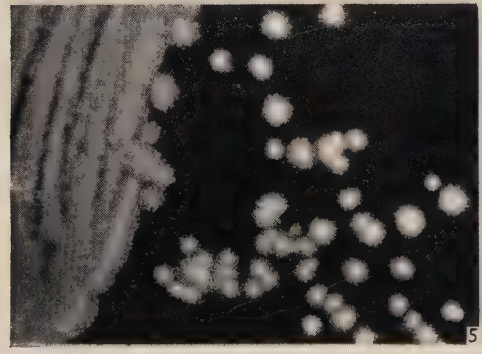

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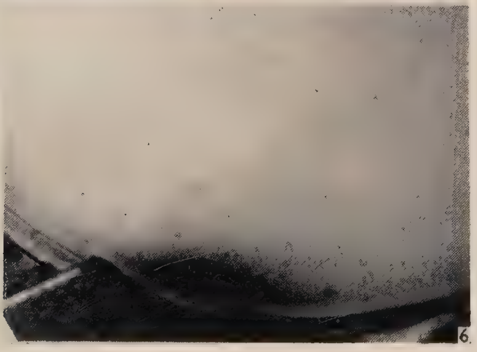

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E. W. McCloy—Lysogenicity and immunity. Plate 1

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The Effect of Inorganic Ions on Respiration in *Pasteurella tularensis* and *Escherichia coli*

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SUMMARY: The effect of the ionic composition of the medium on the respiration of resting-cell suspensions of two Gram-negative micro-organisms has been investigated. Potassium or rubidium salts with Cl^-, Br^- or I^- as anion were found to be necessary for the maximal rates of oxidation of glutamate or glucose by *Pasteurella tularensis*, and KCl or K_2SO_4 by *Escherichia coli*. The pH value of the medium and the concentration of the substrate influenced the effect of the electrolytes. Under certain conditions, a synergistic effect of Na^+ ion was observed. The same ion mixtures which supported the respiration caused the maximal swelling of the bacterial cells.

The influence of KCl and NaCl on the respiration of halophilic bacteria was investigated by Baxter & Gibbons (1956). Krebs, Whittam & Hems (1957) showed that the oxidation of various substrates by washed cells of *Alcaligenes faecalis* was greatly accelerated by potassium ions. In the present communication observations are reported on the effect of various electrolytes on the rate of oxidation of glutamate and glucose by *Escherichia coli* and by an osmotically-sensitive micro-organism, *Pasteurella tularensis*.

METHODS

Escherichia coli, strain B, was grown on nutrient agar (Difco). *Pasteurella tularensis*, strain S and D, was grown on a 3 % (w/v) peptone (Difco) + cysteine medium (Mager, Kindler & Grossowicz, 1954). The cultures were incubated at 37° for 24 hr. The bacteria were collected in 0·25 M-sucrose and, unless otherwise stated, washed three times with this sucrose solution. The final sucrose (0·25 M) suspensions contained 50 mg. wet weight organisms/ml. Manometric experiments were carried out by the conventional Warburg technique. Spectrophotometric examinations were performed in the Beckman model DU spectrophotometer, with Corex cells of 1 cm. light path.

RESULTS

Effect of various cations and anions on the oxidation of glutamate

Glutamic acid, neutralized by the addition of tris (buffer), 2-amino-2-hydroxymethylpropane-1:3-diol, to pH 7·4, was not oxidized by thoroughly washed *Pasteurella tularensis* when other salts were excluded from the reaction medium. When, however, various potassium salts were added to the bacteria suspended in isotonic sucrose, it was found that KBr caused the greatest respiratory stimulation, while KCl and KI were slightly less effective; other potassium salts had only an insignificant effect. Of the chlorides tested other than KCl only the rubidium salt supported the respiration (Table 1). Since KCl and the other effective electrolytes enhanced the respiration at relatively low concentrations, their effect could not be due to changes in osmotic pressure (Fig. 1).

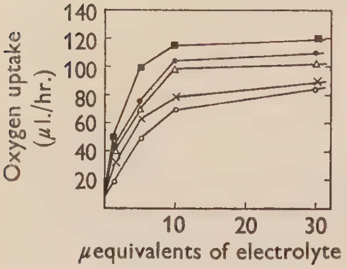

Fig. 1

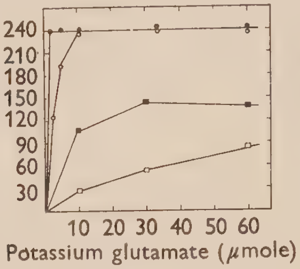

Fig. 2

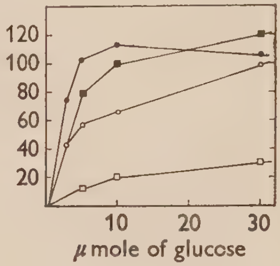

Fig. 3

Fig. 1. Effect of electrolyte concentration. Each flask contained 30µmole tris glutamate buffer (pH 7·4), 0·25M-sucrose, the bacterial suspension (*Pasteurella tularensis*: 15 mg. wet weight; *Escherichia coli*: 9 mg. wet weight) and the electrolyte as indicated. ○–○ and ●–● = KCl; △–△ = KBr; ×–× = KI; ■–■ = K_2SO_4. Unshaded marks; and ×–× : *Pasteurella tularensis*. Shaded marks: *Escherichia coli*. Volume of reaction mixture 3·0 ml.; centre well, 0·2 ml. of KOH. Temperature, 28°; gas phase, air.

Fig. 2. Effect of concentration of potassium glutamate. Each flask contained 0·25M-sucrose, the bacterial suspension as in Fig. 1, and potassium glutamate as indicated. ○–○ and ●–● = *Escherichia coli*; □–□ and ■–■ = *Pasteruella tularensis*. Unshaded marks: no KCl added; shaded marks: 30µmole KCl added. Conditions of experiment otherwise as in Fig. 1.

Fig. 3. Effect of KCl on the oxidation of glucose. Each flask contained 60µmole tris nitrate buffer (pH 7·4) 0·25M-sucrose and glucose as indicated. *Escherichia coli* and *Pasteurella tularensis* as in Fig. 1. ○–○ and ●–● = *Escherichia coli*; □–□ and ■–■ = *Pasteurella tularensis*. Unshaded marks: no KCl added; shaded marks; 30µmole KCl added. The conditions of the experiment otherwise as in Fig. 1.

Escherichia coli likewise was incapable of metabolizing glutamate neutralized by tris (buffer); addition of KCl or K_2SO_4 was necessary for oxidation of the substrate, but rubidium could not replace the potassium (Table 1, Fig. 1). In order to elucidate the part played by the cation, the rate of oxidation of glutamate in various counter-ion combinations was measured. Sodium glutamate was not oxidized by either of the two micro-organisms. In *Pasteurella tularensis*, the rate of oxidation of potassium glutamate increased with the concentration of the substrate, and the highest rate of respiration was attained only when KCl was added. In suspensions of *Escherichia coli*, KCl accelerated the oxidation of potassium glutamate only when the concentration of the substrate was suboptimal (Fig. 2).

Effect of KCl *on the oxidation of glucose*

Acceleration of the rate of glucose oxidation by the addition of KCl was also demonstrated. In *Pasteurella tularensis*, the effect was observed at all concentrations of substrate, while in *Escherichia coli* it became apparent only at low glucose concentrations (Fig. 3).

Effect of pH *value*

The effect of the pH value on the oxidation of glutamate and glucose was studied in *Pasteurella tularensis*. As shown in Fig. 4, the rate of oxidation of both substrates diminished with decreasing acidity. The stimulation caused by KCl became most prominent at slightly alkaline pH values.

Table 1. *The effect of electrolytes on the oxidation of tris glutamate buffer by* Escherichia coli *and* Pasteurella tularensis

Each flask contained 30 μmole of tris glutamate buffer (pH 7·4), 0·25 M-sucrose. The final concentration of the electrolytes was 30 μequiv./cup when one salt was added and 15 μequiv./cup each salt when a mixture of two was used. Volume of reaction mixture 3·0 ml.

	Cation							Organism	
								E. coli	*P. tularensis*
								Oxygen uptake (μl./hr.)	
Anion	K^+	Na^+	Li^+	Rb^+	NH_4^+	Ca^{++}	Mg^{++}		
Chloride	+	.	.	.	.	.	.	186	120
	.	+	.	.	.	.	.	60	17
	.	.	+	.	.	.	.	0	45
	.	.	.	+	.	.	.	0	142
	.	.	.	.	+	.	.	0	0
	.	.	.	.	.	+	.	40	0
	.	.	.	.	.	.	+	0	42
Bromide	+	.	.	.	.	.	.	10	154
	.	+	.	.	.	.	.	0	.
	+	+	.	.	.	.	.	138	.
Iodide	+	.	.	.	.	.	.	15	106
Nitrate	+	.	.	.	.	.	.	0	0
	.	+	.	.	.	.	.	12	0
	.	.	+	.	.	.	.	0	.
	.	.	.	.	.	+	.	0	.
	.	.	.	.	.	.	+	0	.
	+	+	.	.	.	.	.	185	34
	+	.	+	.	.	.	.	0	.
	+	.	.	.	.	.	+	0	.
	+	.	.	.	.	+	.	0	.
Sulphate	+	.	.	.	.	.	.	185	35
	.	+	.	.	.	.	.	0	20
	+	+	.	.	.	.	.	147	20
Phosphate	+	.	.	.	.	.	.	0	47
	.	+	.	.	.	.	.	0	.
	+	+	.	.	.	.	.	153	.
	.	.	.	.	.	.	.	0	0

Controls containing each electrolyte but no glutamate showed no oxygen uptake.

The synergistic effect of Na^+ *ions*

The suppression of the oxidation of glutamate in *Escherichia coli* in the presence of potassium salts other than chloride and sulphate (i.e. NO_3^-, HPO_4^-, etc.) can be counteracted by the addition of Na^+ ions, irrespective of the anionic moiety (Table 1). Li^+, Rb^+, Mg^{++} or Ca^{++} could not replace Na^+ in this respect.

Effect of electrolytes on changes in the state of swelling of bacterial cells

When a suspension of *Pasteurella tularensis* was added to a medium containing KCl alone, or glutamate neutralized with tris (buffer), only slow swelling was observed. In a mixture of the substrate + the electrolyte, however, the optical density of the suspension decreased rapidly and exponentially until a new state of equilibrium was reached. Like KCl, KBr and KI accelerated swelling but other potassium salts had no effect (Fig. 5). In an experiment with a suspension of *Escherichia coli*, KCl, K_2SO_4 or a mixture of $KNO_3 + NaNO_3$ was found to promote swelling.

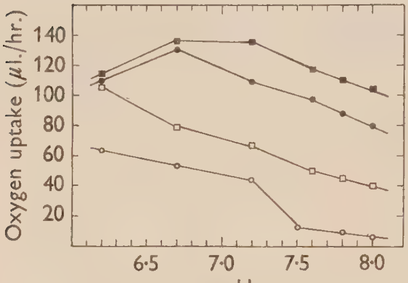

Fig. 4

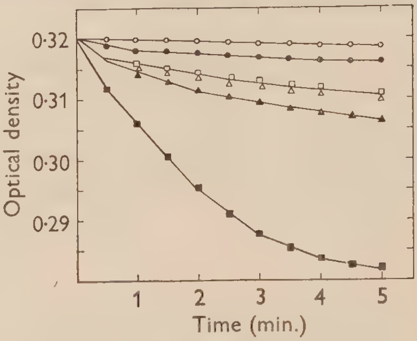

Fig. 5

Fig. 4. Effect of pH value. Each flask contained: 60 μmole tris phosphate buffer of the respective pH value; 30 μmole substrate; 0·25 M-sucrose; *Pasteurella tularensis* as in Fig. 1. ○–○ and ●–● = glutamate; □–□ and ■–■ = glucose. Unshaded marks: no KCl added; shaded marks 30 μmole KCl added. Conditions of the experiment otherwise as in Fig. 1.

Fig. 5. Effect of glutamate and electrolytes on turbidity changes. Each Beckman cell contained: 0·25 M-sucrose and tris glutamate buffer and/or electrolytes as indicated. ○–○ = no addition; ●–● = 30 μmole tris glutamate buffer; □–□ = 30 μmole KCl; △–△ = 30 μmole KNO_3; ■–■ = 30 μmole glutamate + KCl; ▲–▲ = 30 μmole glutamate + KNO_3. Total volume 3·0 ml. At zero time 0·05 ml. of a dense suspension of *Pasteurella tularensis* added. Control cell, water. Room temperature. Optical density measured at wavelength 500 mμ.

DISCUSSION

The effect of electrolytes on the respiration of a variety of biological systems has been described before. Acceleration of the respiration of brain cortex slices by chlorides, bromides or iodides of either potassium, rubidium or caesium has been demonstrated (Ashford & Dixon, 1935; Dickens & Greville, 1935). The stimulation of glucose oxidation in rat-liver homogenates by K^+, Rb^+, Cs^+, Mg^{++} was reported by Haugaard & Itzkovitz (1956); on addition of NaCl, LiCl or $CaCl_2$ to a system activated by potassium ions, glucose oxidation was inhibited. In *Alcaligenes faecalis* washed with distilled water, Krebs *et al.* (1957) demonstrated an acceleration of the rate of oxidation of various substrates by the addition of K^+ or Rb^+ ions. These authors attributed the activity of Rb^+ to contamination with potassium salts. In the present work, the same batch of rubidium chloride which replaced K^+ in *Pasteurella tularensis* proved to be quite ineffective on the oxidation of glutamate in *Escherichia coli*. In this case, therefore, the action of Rb^+ cannot be attributed to small amounts of K^+ present as impurity. In *Alcaligenes faecalis*, accumulation of K^+ ions was shown to occur during the incubation and could be accounted for by exchange diffusion. The penetration of K^+ was not accompanied by a similar movement of the anion, and moreover the nature of the anion was without influence on the rate of acceleration of the respiration. In contrast to these findings, K^+ ion was effective only in certain counter-ion combinations in *Escherichia coli* and *Pasteurella tularensis*. Exchange diffusion seems, therefore, to play a smaller part in this case. It might be relevant to this point that, in the case of *Escherichia coli*, potassium salts which alone caused no stimulation of the respiration, could be activated by the addition of any sodium salt which by itself was similarly ineffective. A promoting effect on the exchange diffusion of potassium by sodium ions, overcoming thereby the retardation due to the anion, might be involved in this phenomenon. The control of the influx of K^+ ions by the Na^+ concentration in the medium into erythrocytes and nerve fibres has repeatedly been demonstrated (Conway, 1954). Krebs *et al.* (1957) have also shown that Na^+ ions facilitate the accumulation of K^+ in the bacterial cell.

As potassium glutamate is oxidized by *Escherichia coli* at maximum rate without the addition of other electrolytes, the anion has probably only an indirect effect. The anion might exert a modifying action on the permeability of the cation, due to the different diffusion potentials created (Davson, 1940). The effectiveness of the halogen salts of potassium and rubidium in *Pasteurella tularensis* is in accordance with this view, as biological membranes are known to be permeable to Cl^-, Br^- and I^- ions (Conway, 1954). The penetration of the cation used as its halogen salt will thus not be hindered by its counter-ion. The ready penetration of the sulphate ion in *Escherichia coli*, on the other hand, could not be anticipated.

Correlation between the swelling of ascites cells suspended in media containing various substrates and the accumulation of the metabolites in the cell interior has been demonstrated before (Christensen, Riggs, Fisher & Palatine, 1952). The finding that the same ion combination, which accelerated the oxidation of substrates in the micro-organisms studied, was also the most effective in eliciting the swelling of the bacteria (Mager, Kuczynski, Schatzberg & Avi-Dor, 1956; Avi-Dor, Kuczynski, Schatzberg & Mager, 1956) could possibly be interpreted as the effect of the ionic environment on the rate of penetration of the substrate. The latter might become the rate limiting step under certain experimental conditions (higher pH values, low substrate concentration). Hence, the increased dependence of the respiration on the addition of K^+ ions under such conditions.

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The Unilinear Transmission of Motility and its Material Basis in Salmonella

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SUMMARY: Certain sub-lines of a strain of *Salmonella paratyphi* C contain 10 % or more of motile flagellated bacteria when in exponential growth at 37°. Less than 0·1 % of the organisms are motile after prolonged exponential growth at 20°. Single motile organisms isolated from cultures grown at 37° transmitted motility to only one or a few of their descendants during growth at 20°; they behaved as if their motility were due to non-multiplying motility-conferring (MC) particles. There is a good correlation between the distributions of number of MC particles/motile bacterium as indicated by the numbers of motile descendants at 20°, and the distributions of number of flagella/flagellated bacterium in stained preparations. It is inferred that one MC particle corresponds to one flagellum. The results support the hypotheses that in some cases flagella arise as a consequence of discontinuous intracellular events, each of which leads to the synthesis of a small number of flagella, and that parental flagella are shared amongst progeny bacteria until no descendant has more than one flagellum derived from a parental cell several generations removed. This sharing is not detected unless synthesis of new flagella ceases whilst bacterial growth continues. Single flagella may be unilinearly transmitted for over 20 generations.

During experiments on the transduction of motility in Salmonella, Stocker (1956*a*, *b*) and Lederberg (1956) encountered a class of motile bacterium which divided to produce one non-motile and one motile daughter. The motile daughter in its turn behaved similarly; the character 'motility' was transmitted down a single line of descent to one only of its progeny of any generation. Stocker (1956*a*, *b*) called this phenomenon the *unilinear transmission* of motility and to account for it he put forward the hypothesis of motility-conferring (MC) particles. The hypothetical MC particles have the following properties:

(1) A single particle enables the bacterium bearing it to swim in broth, but several particles are required to enable the bacterium to swim through a semi-solid medium.

(2) A particle is not replicated, but is retained by one of the daughter cells at division.

(3) A particle, and in consequence motility, can be handed on for many generations, e.g. 20.

(4) When a bacterium bearing several MC particles divides, the particles are shared about equally between the daughters.

The material basis for the hypothetical MC particles is not clear. The unilinear transmission of motility entails the transmission of effective locomotor apparatus: possibly a single flagellum or a basal granule which synthesizes it, a whole cell wall bearing one or more flagella, or some 'indivisible' intracellular mechanism which controls the synthesis of flagella. Motile bacteria to which motility has been unilinearly transmitted (and thus by definition having only one MC particle each) are readily isolated by micromanipulation. Unfortunately, it was not possible to test whether each had only one, or several, flagella, since a single unstained flagellum of Salmonella is invisible in the light microscope and the staining of selected individual bacteria is not feasible.

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Unilinear transmission of motility was also encountered by Lederberg (1956) and by Quadling & Stocker (1957) when investigating the behaviour of rare, non-mutant, motile bacteria which they found occurring spontaneously in some non-motile Salmonella strains. Stocker (1956*b*) suggested that unilinear transmission might be detectable whenever motile bacteria multiply to produce predominantly non-motile offspring, i.e. when multiplication continues but inception of *new* flagella ceases. This situation has now been achieved experimentally by transferring exponentially-growing cultures of certain Salmonella strains from 37°, at which temperature a high proportion of the bacteria are motile, to 20°, at which temperature bacterial growth continues but synthesis of new flagella ceases. Such experiments have enabled comparisons to be made between distributions of number of motile progeny/motile bacterium (i.e. by definition, of number of MC particles/motile bacterium) and the distributions of number of stained flagella/flagellated bacterium.

There are numerous reports of bacteria which are motile when grown at one temperature and non-motile when grown at other, usually higher, temperatures. Reports of better development of motility at high temperatures of incubation are rare, e.g. Neustadl (1917). The *Salmonella paratyphi* C strains used in the present work (all derived from the same O parent strain) are unusual in that flagellation and motility are best developed at high incubation temperature (37°).

METHODS

Organisms and media. The cultural methods and media used were as described by Quadling & Stocker (1957). Cultures were grown either at 37°, or at laboratory temperature (*c.* 20°); such cultures are hereafter termed '37° cultures' and '20° cultures'.

The *Salmonella paratyphi* C O strain of Hayes & Freeman (1946), hereafter termed SL 236, was received from Dr C. C. Spicer (Serological Standards Laboratory, Public Health Laboratory Service, Colindale, London, N.W. 9); this strain was non-motile and non-flagellated when grown at 20° and 37° in any medium tested, and it did not spread in semi-solid agar. A motile mutant, SL 237, was selected from it by picking from a slow-spreading swarm produced in semi-solid medium incubated at 37°. When SL 237 was grown at 20°, in tryptic- or papain-digest broth, less than 0·1 % of the bacteria were motile and less than 0·1 % were flagellated; but when grown at 37° in tryptic-digest broth about 15 %, and in papain-digest broth about 70 %, of the bacteria were flagellated and motile. A further substrain, hereafter referred to as SL 242, was obtained from SL 237 by isolation from a fast-spreading swarm produced in semi-solid medium. SL 242 also was non-motile and non-flagellated when grown at 20°; when grown at 37° in tryptic broth about 40 % of the bacteria were flagellated and motile. The proportion of motile bacteria in 37° cultures of SL 237 and SL 242 varied somewhat from experiment to experiment, presumably as a result of differences between batches of broth. Strains designated by a strain no. followed by a capital letter, e.g. SL 237/A, were sublines, each grown from a single bacterium isolated by micro-manipulation from the parent culture.

Staining and counting of flagella. A modification of Leifson's (1951) method was used to stain flagella on bacteria grown in broth. Cultures were fixed with formaldehyde, to a final concentration of 1 % (w/v), then sedimented by centrifugation for 40 min. at 250 **g**. The bacteria were then washed twice by gentle resuspension in 0·01 % (v/v) Teepol (Shell Chemicals Ltd., 105/109 Strand, London, W.C. 2) in distilled water and recentrifuged. The bacteria were finally resuspended in distilled water. The detergent was used to ensure the uniform distribution of organisms in smears (Snieszko, 1942) and to assist in attaining the cleanliness necessary with this staining technique. Drops of the final distilled-water suspension of organisms were transferred to acid-cleaned slides and stained as described in Leifson (1951).

Bacteria were usually well dispersed in the central part of each smear; counts of number of flagella/bacterium were made on bacteria in this central region. Counts were made at a magnification of × 1200 by using a 2 mm., × 95 oil-immersion objective with phase-contrast illumination and a green filter. The flagella were counted on each of the first five flagellated organisms seen in each of ten or more successive scans started at arbitrarily chosen points. The use of phase-contrast made counter-staining of the bacterial bodies unnecessary.

Micromanipulation. The techniques used followed those of de Fonbrune (1949) as described by Stocker (1956*b*) and Quadling & Stocker (1957).

RESULTS

Demonstration of unilinear transmission of motility

When strain SL 237/A was grown at 37° in tryptic-digest broth about 15 % of the bacteria were flagellated and about 15 % were motile; when grown at 20° the proportion of motile bacteria was very low ($<0·1$ %) and in stained preparations the proportion of flagellated bacteria was similar. Motile bacteria were isolated by micromanipulation from 37° cultures and were allowed to continue growth at room temperature (*c.* 20°) in microdrops in the oil chamber. Under these conditions each motile bacterium, after seven to eight divisions, gave rise to a predominantly non-motile population containing only one or a few (up to six) motile bacteria (Table 1). Non-motile bacteria when isolated produced only non-motile populations.

Evidence for the unilinear transmission of motility was obtained by making repeated isolations of motile descendants during growth at 20° of populations each descended from a motile bacterium originally isolated from a 37° culture. A typical experiment is illustrated in Fig. 1. In the lineage illustrated a motile bacterium, isolated from a 37° culture of strain SL 237/A, divided at 20° to give three motile bacteria and one non-motile bacterium. Each of these bacteria was isolated to a sterile drop of broth, where growth continued. The populations were examined at intervals and any motile bacteria present were removed to fresh drops. At no time were there more than three motile bacteria amongst the descendants of the bacterium originally isolated. On the hypothesis that a bacterium which transmits motility unilinearly has one MC particle and that one MC particle corresponds to one flagellum, the original motile bacterium had three flagella; these were shared between the daughters at the first two divisions after isolation and persisted for up to twenty generations amongst the descendants. Similar results were obtained with motile bacteria isolated from 37° cultures of strain SL 242/A.

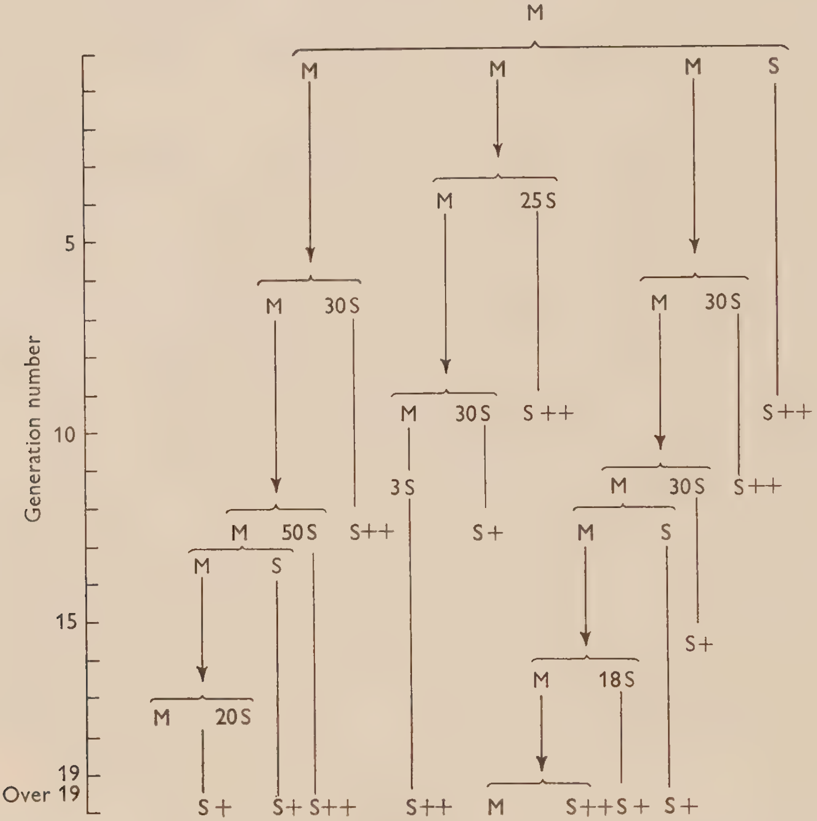

Fig. 1. **Isolation by micromanipulation of the motile progeny of a motile bacterium from a culture of strain SL 237/A grown in tryptic-digest broth at 37°. The progeny were grown at 20° in microcultures. Arrows indicate transfer of a motile bacterium to a sterile microdrop. M = a motile bacterium; S = a non-motile bacterium; + = hundreds; + + = thousands.**

The motile bacteria isolated from 37° cultures of these two strains behave at 20° like one class of motile bacteria produced as a result of abortive transduction of motility (Stocker, 1956*b*; Lederberg, 1956) or like motile bacteria occurring spontaneously in certain non-motile strains (Quadling & Stocker, 1957). That is, they transmit motility only to a few of their descendants, as if their motility were due to the presence of a few non-multiplying MC particles whose further synthesis, in the present case, ceased on transfer to 20°, and as if the MC particles were transmitted to correspondingly few of the progeny of the originally isolated motile bacterium.

Distributions of numbers of motile descendants and of numbers of stained flagella amongst the bacteria

If an MC particle corresponds to a flagellum, then a bacterium (of a 37° culture) with n flagella when allowed to multiply at 20° for several generations would have n motile descendants; and the proportions of bacteria of a 37° culture having 1, 2, 3, etc. flagella should be equal to the proportions having 1, 2, 3, etc. motile descendants at 20°. To determine the frequency distribution of flagella/bacterium, and of motile descendants/bacterium, cultures were first kept in logarithmic growth at 37° for several hours, to enable the population to reach a steady-state composition in respect of motility and flagellation. Culture samples were then placed in the microdissection chamber at 20°; motile bacteria were picked and isolated to individual microdrops of broth. When the populations of the microcultures numbered about 250, the motile bacteria in each drop were counted.

Samples of the same 37° cultures, from which motile bacteria had been isolated, were fixed with formalin, and the distributions of number of flagella/flagellated bacterium determined. Determinations of distributions of number of flagella and of number of motile descendants were made on 37° cultures of SL237 grown in tryptic-digest broth and in papain-digest broth, and on 37° cultures of SL242 grown in tryptic-digest broth (the proportion of flagellated, motile, bacteria in the three cases being respectively *c*. 15 %, *c*. 70 % and *c*. 40 %); results are given in Table 1 and are illustrated by histograms in Fig. 2.

Some possible errors

The observed distributions of numbers of motile descendants and by inference of numbers of MC particles are subject to at least three sources of error. These are: (1) failure to detect some motile bacteria in non-motile microcultures; (2) unconscious selection of more highly motile bacteria for isolation; (3) changes in the composition of cultures (*a propos* of distributions of numbers of MC particles) during the period in which motile bacteria were being isolated.

The efficiency of detection of motile bacteria in micromanipulation experiments was discussed by Stocker (1956*b*) and by Quadling & Stocker (1957). The evidence (e.g. Fig. 1) of repeated detection of motile bacteria over a number of generations indicates that the efficiency of detection was high in the experiments

Table 1. *Distributions* of numbers of flagella/flagellated bacterium and of numbers of motile descendants/motile bacterium in 37° cultures*

Culture	No. of flagella or of motile descendants...	*0*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	No. of bacteria scored	Proportion flagellated and motile in whole culture
SL 237/A, tryptic-digest broth	Flagella	·	79	16	4	1	·	·	·	·	·	·	·	·	100	c. 15 %
	Motile descendants	0	56·9	23·2	7·7	7·7	1·5	1·5	0	0	0	1·5	·	·	65	
SL 237/A, papain-digest broth	Flagella	·	48	26	14	11	0	1	·	·	·	·	·	·	100	c. 70 %
	Motile descendants	8·2	33·7	20·4	15·3	6·1	3·1	3·1	3·1	2·0	2·0	0	1·0	2·0	98	
SL 242/A, tryptic-digest broth	Flagella	·	34	27	22	9	5	2	0	1	·	·	·	·	100	c. 40 %
	Motile descendants	0	24·3	18·6	12·9	10·0	14·3	8·6	2·9	4·3	2·9	0	1·4	·	70	

* Proportions of bacteria with indicated number of flagella or motile descendants are stated, respectively, as % of flagellated or of motile bacteria observed.

described here. Errors of the second category might lead to distortion of the distributions if such highly motile bacteria produced larger numbers of motile descendants than less motile bacteria. However, in the strains used here it was impossible to predict, on the basis of swimming behaviour in broth, whether any given motile bacterium would give rise to one or several motile descendants, i.e. would prove to have had one or several MC particles. Bias of this type is therefore considered improbable.

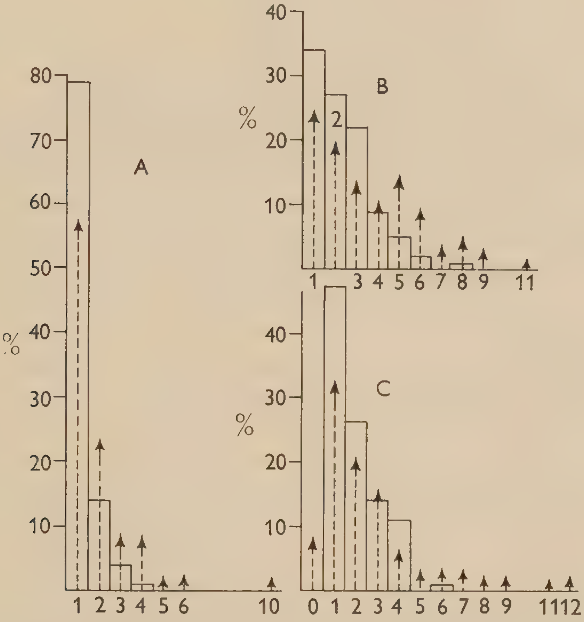

Fig. 2. Histograms constructed from the data of Table 2. Solid columns record % of flagellated bacteria having the indicated number of stained flagella. Arrows record % of motile bacteria having the indicated number of motile descendants at 20°. (A) SL 237/A grown at 37° in tryptic-digest broth. (B) SL 242/A in tryptic-digest broth. (C) SL 237/A in papain-digest broth.

Errors of the third category are *ex hypothesi* unavoidable: motile bacteria may give rise to small numbers of motile progeny in the course of their first few divisions after isolation (e.g. Fig. 1); thereafter no increase occurs in the number of motile descendants. This is economically interpreted as being due to the sharing of parental MC particles between daughters. Such sharing during the continued growth of 37°-grown cultures at 20° would result in a decrease in average number of MC particles/motile bacterium. To minimize this error a maximum period of 30 min. was allowed between the removal of a culture sample from 37° and completion of isolations of motile bacteria at 20°; this represents about three-eighths of a mean generation-time of these organisms at 20°.

The observed distribution of numbers of stained flagella may be inaccurate for at least two reasons. Organisms may be mechanically deflagellated during preparation for staining and precautions were taken to minimize this error;

control experiments, in which organisms were prepared for staining by dialysis rather than centrifugation, indicated that a decrease not greater than 20 % in mean number of flagella/bacterium might occur during preparation. A second inaccuracy arises from the tendency of flagella to aggregate into bundles (see Stocker 1956*a*); in a heavily stained preparation a bundle of several flagella might appear as one unit. This error was minimized by using lightly stained preparations.

DISCUSSION

When comparing the distributions of number of motile descendants with those of the number of flagella it must be noted that the former refers only to bacteria selected for isolation because of their motility and the latter only to those bacteria which possessed at least one stainable flagellum. The % of motile bacteria did not differ detectably from the proportion of flagellated bacteria; presumably all or nearly all flagellated bacteria were motile and all or nearly all motile bacteria were detected as flagellated in the stained preparations. If these assumptions are justified the two distributions are based on the same population, namely, that fraction of the culture which was flagellated and motile at the time of sampling.

Even if parental flagella are shared amongst progeny and are thereafter transmitted unilinearly with each single flagellum rendering its possessor motile (i.e. even if the single flagellum is the postulated MC particle), exact coincidence of the distributions of number of flagella and of motile descendants/bacterium is not to be expected in a growing culture in a steady-state in respect of synthesis of flagella. Distributions of numbers of stained flagella represent the condition of the culture at the instant of treatment with formaldehyde; presumably developing flagella already in process of extrusion will not be detected if shorter than some critical value. Distributions of numbers of motile descendants comprise bacteria motile by means of flagella which were functional at the time of isolation and also descendant bacteria motile by virtue of flagella which completed their development subsequent to the time of isolation. Presumably flagella which were in course of development when the motile organisms were transferred to 20° will, after further development, confer motility on the progeny which 'inherit' them. Thus, mean numbers of motile descendants/motile organism would be expected to exceed the corresponding mean numbers of stained flagella/flagellated organism. The ratios of these mean numbers (Table 2) for each pair of comparable distributions (average 1·3:1) are in reasonable agreement in the three cases. The significance of the indicated predominance of motile descendants is not entirely clear however. That the inception of new flagella ceases, or proceeds at greatly diminished 'residual' frequency, when populations of these strains are transferred from 37° to 20° is indicated by their gradual transition to almost complete (more than 99·9 %) non-motility during growth at the lower temperature. It is not known whether such inception ceases abruptly or decreases gradually after the temperature change. Increase in numbers of motile descendants due to such residual synthesis is likely to be small compared with that due to completion of development by flagella already in process of extrusion at the time of temperature change.

It is concluded from the general similarities of comparable distributions that a correlation exists in these strains between the number of flagella borne by an organism and the number of motile descendants to which it can give rise under conditions in which flagella inception, but not growth, has ceased. The simplest explanation for this correlation is that each motile descendant receives one of the flagella of the originally-isolated bacterium, so that a bacterium with, for example, three flagella gives rise to three motile descendants after several generations of growth. This implies that parental flagella are shared among progeny bacteria in such a way that after several generations of fission, in the absence of inception of new flagella, each motile descendant will have no more than one flagellum. Further evidence that parental flagella are shared amongst progeny bacteria, in the manner indicated above, was reported by Quadling & Stocker (1956).

Table 2. *Summary of results on the correspondence between stainable flagella and numbers of motile descendants*

All cultures were initially grown at 37°; motile descendants were counted when populations derived from single motile bacteria had grown to 100–500 at 20°. The flagella in samples of 37°-grown cultures were stained by Leifson's (1951) method.

Strain	Bacteria flagellated and motile (%)	Mean no. motile descendants/motile bacterium		Mean no. flagella/ flagellated bacterium	Ratio: mean no. motile descendants: mean no. flagella	
		Observed	Adjusted ×1·2*		Observed	Adjusted ×1·2*
SL 237/A†	*c.* 15	1·59	1·91	1·27	1·25	1·50
SL 237/A‡	*c.* 70	2·58	3·10	1·91	1·35	1·62
SL 242/A†	*c.* 40	3·30	3·96	2·37	1·39	1·67
Pooled data	—	—	—	—	1·33	1·60

* Observed mean adjusted by factor of ×1·2 to allow for estimated extent of 'dilution-out' of flagella during the period 0–30 min. in which isolations were made.

† Culture grown in tryptic-digest broth.

‡ Culture grown in papain-digest broth.

The conclusions detailed above conflict with those reached by Bisset (1951) and by Bisset & Pease (1957). These authors inferred from electron micrographs and stained preparations that in dividing Gram-negative bacteria the portion of the cell wall bearing the parental flagella was retained in its entirety by one daughter, the other daughter growing new flagella. If parental flagella are partitioned in this 'all or nothing' manner, in order to account for the results presented here it is necessary to postulate that there is a gradual loss of ability to synthesize new flagella amongst the immediate progeny of the originally isolated motile bacteria, so that, for example, in the case of a bacterium which gives rise to four motile descendants synthesis of new flagella continues for two generations after transfer to 20°. Furthermore, the length of time at 20° taken for this loss to occur in a particular strain grown in a particular medium would have to be correlated with the numbers of flagella originally borne by the organisms. Whilst this interpretation cannot be excluded, the conclusions in the preceding paragraph are preferable on the grounds of economy of hypothesis.

If these conclusions are correct, the observed distributions of number of flagella/bacterium can be regarded as a product of (1) growth, and division, which result in the sharing-out of already-present flagella, and (2) the frequency and characteristics of intracellular 'events' which lead to the synthesis of new flagella. The event hypothesis was advanced by Quadling & Stocker (1957) to describe the infrequent synthesis of MC particles in 'bursts' of small numbers during growth of bacteria of some non-flagellated Salmonella strains; an event was regarded as a transient change in cell phenotype to the flagella-synthesizing state. The large proportion of non-flagellated bacteria in the cultures used in the present work indicated that, even at 37°, only a proportion of the bacteria synthesize flagella at any one time. Presumably events analogous to those described by Quadling & Stocker (1957) occur also in these strains. When events occur with low frequency the flagella generated during an event will be shared amongst the progeny so that each will tend to have no more than one flagellum by the time a fresh event supervenes. The condition will then be reached in which bacteria with 0, 1, 2, . . ., n (where n is the maximum observed number of flagella produced per event) are characteristically present in the culture, as described for the strains studied here. Cultures presenting this appearance have been said to show 'degenerate peritrichous flagellation' (Conn & Wolfe, 1938).

In general, the results reported here support the hypothesis that an MC particle is equivalent to a flagellum and that MC particles (flagella) are produced in bursts of small numbers. They also provide further evidence of non-genetic heterogeneity *à propos* of flagellation and motility which occurs in clonal bacterial populations growing in a common (liquid) environment; such overt heterogeneity is presumably indicative of underlying biochemical heterogeneity.

I wish to thank Dr B. A. D. Stocker for help and encouragement freely given at all stages of this work. It is also a pleasure to thank Dr P. H. A. Sneath for helpful discussion of flagella-staining technique. This paper is based on part of a Ph.D. thesis submitted to the University of London, 1956.

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Teepol in Substitution for Bile Salts

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SUMMARY: When any grade of Teepol, other than Teepol 530, Teepol L or Teepol (without suffix), is used in culture media, the authors recommend that it should first be tested for suitability.

The use of Teepol, a modern anionic detergent marketed by the Shell Chemical Company Ltd., was described by Jameson & Emberley (1956) as a substitute for bile salts in culture media.

In July 1957 the authors again wrote to the Shell Chemical Company and asked whether it would be possible for an assurance to be given that no product differing materially in composition from that previously supplied to them would in the future be marketed under the trade name of Teepol. In their reply the manufacturers stated that formulations incorporating various types of surface active agents were marketed under the Teepol brand name and the active ingredients of these were not all identical chemically. Teepol 530 was the brand originally used by Jameson & Emberley (1956). Teepol L, marketed through British Drug Houses Ltd., is of similar chemical constitution as is also the material marketed in the U.K. for general cleaning purposes under the Teepol brand without suffix. Other brands of Teepol with suffixes different from those enumerated above may be based on different surface active agents or contain additives and should, therefore, be tested for suitability before use in the manner described by Jameson & Emberley (1956).

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Preliminary Studies on the Inoculation of Selected Micro-Organisms into Partially-Sterilized Soils

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SUMMARY: Samples of Rothamsted and Woburn soil were either partially sterilized, totally sterilized or untreated. Representative samples of each treatment were inoculated with a pure culture of *Nocardia cellulans* and other samples, uninoculated, were kept as controls. The change in numbers of nocardias/g. soil was followed at intervals for one year. In partially-sterilized soil wide fluctuations occurred during the first month, followed by a steadying in count, and at the end of the year *N. cellulans* was still present in high numbers. The numbers of bacteria in such a soil were depressed in comparison with the uninoculated control. *N. cellulans* disappeared from an untreated soil in 6 months. The wide fluctuations in numbers in partially sterilized soil were correlated with a regular cyclical morphological development of *N. cellulans*. This was demonstrated by morphological observations and a soil-shaking method. Peak counts corresponded to the rod form and troughs to the mycelial form and the steadying in count to the rod form.

After soil is partially sterilized by heat or fumigation the number of micro-organisms decreases rapidly for the first few days, then increases during the next few weeks to a number exceeding that in an untreated soil and finally falls again to a number similar to that in control soils (Hall & Clegg, 1949). Usually such partially-sterilized soils are at first very favourable to colonization by fungi, including root-disease fungi, because nutrients are liberated and competition is temporarily limited. With further colonization, however, such soils may become less favourable to parasitic activity than the original unsterilized soils, depending on the type of microflora that establishes itself after soil treatment (Garrett, 1956). Ludwig & Henry (1943) found that *Ophiobolus graminis* re-introduced into a steamed soil was less virulent than when introduced into untreated soil. They attributed this to the activity of *Trichoderma viride* which had become established in the steamed soil.

Inoculation of partially-sterilized soils with micro-organisms selected for their antagonistic action towards plant pathogens was recommended by van Luijk (1938; quoted in Waksman, 1947, p. 306) as a method for controlling plant disease. Hartley (1921) also suggested that partially-sterilized soil could be re-inoculated with a mixture of saprophytic micro-organisms before using it as a seed-bed, so as to counterbalance the injurious effects of parasites. Such inoculations as these have only a temporary effect in changing the biological balance of the soil population. However, if the organisms could be permanently established, then the inoculation method might become a more effective means of controlling disease.

The present work was undertaken to elucidate some of the factors which affect inoculation into partially-sterilized soils. To follow the changes in population numbers of an introduced culture, an organism which formed easily recognizable colonies was used. *Nocardia cellulans*, a member of the Actinomycetales, which was isolated from chalk soil, can decompose cellulose and can fix atmospheric nitrogen (Metcalfe & Brown, 1957), was selected since as it forms bright yellow colonies on yeast agar.

METHODS

Soil samples. Samples of soil were collected from an area of permanent grassland at Rothamsted and from Stackyard field at Woburn Experimental Station. The Rothamsted soil is a heavy clay-loam mixed with flints (pH 6·8); the Woburn soil is a light sandy soil derived from the Lower Greensand (pH 5·6). The soils were passed through a 3 mm. sieve and known amounts added to previously weighed 500 ml. Erlenmeyer flasks. The flasks were then either partially sterilized by steaming for 1 hr., totally sterilized by autoclaving at 15 lb./sq.in. for 1 hr., or left untreated. Representative samples of soil of each treatment were inoculated with *Nocardia cellulans* and other samples maintained as controls. All samples were incubated at 25° and the moisture content kept constant at 25 % (on dry-weight basis) for Rothamsted soil and at 11 % for Woburn soil, by adding sterile distilled-water when necessary.

Preparation of Nocardia cellulans *inoculum.* The *N. cellulans* inoculum was prepared by adding 1 ml. of a heavy suspension in saline of a 24 hr. culture (grown on yeast agar; (%, w/v): 1·5, yeast; 0·5, peptone; 2·0, agar, pH 7·0) to 1 g. sterilized Vermiculite (made by Pan Brittanica Ltd. Waltham Abbey, Essex) in a screw-capped bottle. The bottle was shaken vigorously to coat the Vermiculite particles with the suspension and the Vermiculite then shaken on to the partially-sterilized soil and well mixed with it, thus ensuring an even distribution of inoculum.

Method of counting Nocardia cellulans *in the inoculated soil samples.* The numbers of *N. cellulans*/g. soil were estimated from twofold dilution series, to 10^{-6}, of the soil made in sterile tap-water. Counts were made by inoculating 16 replicate drops (each of 0·01 ml.) of a suitable soil dilution on to the surface of a yeast agar plate previously dried for 1 hr. The number of colonies which developed within each drop-area was counted after 4 days of incubation at 25°; thus 16 replicate counts were obtained for each dilution. These counts followed a Poisson distribution. The number of *N. cellulans*/g. soil with the standard error was estimated from the total of the 16 counts. Counts were done immediately after inoculation of the soil and at regular intervals during one month, followed by less frequent estimations during one year. In all the experiments significance of differences between numbers of organisms/g. soil was taken at the 5 % level. In addition, counts were made, from the yeast agar plates, of bacteria present in the partially-sterilized soils, with and without the inoculum of *N. cellulans*.

RESULTS

Establishment of Nocardia cellulans *in partially-sterilized soils*

Separate experiments were made on three samples of Rothamsted soil and one with Woburn soil. With Rothamsted soil in Expt. 1, the following sets of soil samples were examined: partially sterilized, inoculated with *Nocardia cellulans* and uninoculated; autoclaved, inoculated; untreated, inoculated. In Expts. 2 and 3 partially-sterilized and inoculated soils were used. With Woburn soil in Expt. 4 partially-sterilized and inoculated soil samples were used.

Following inoculation into partially sterilized Rothamsted soil (Expts. 1–3) the count of *Nocardia cellulans*/g. soil rose steadily for 8–12 days and then fell (Fig. 1). Further wide fluctuations occurred for the next 18 days and over the one-year period the number varied significantly, but never to the same extent as during the first month. At the end of one year counts were still high (Expt. 1: $2\cdot5\times10^8$/g. soil; Expt. 2: $1\cdot7\times10^8$/g.; Expt. 3: $2\cdot7\times10^7$/g.) indicating that *N. cellulans* was competing successfully for available nutrients with the rest of the microbial population in the partially-sterilized soil. These counts were compared with those of *N. cellulans* inoculated into autoclaved and untreated soil (Expt. 1). Similar fluctuations were observed at first in both soils, but in the totally-sterilized soil the count was much higher than in that partially sterilized reaching $5\cdot0\times10^9$/g. soil in 7 days and a maximum of $9\cdot3\times10^9$/g. in 12 days. After 16 days in the untreated soil the count gradually decreased to $4\cdot0\times10^6$/g., a value below that of the initial inoculum of $1\cdot2\times10^7$/g. (Fig. 1). After 6 months, at a dilution of 1/4000 *N. cellulans* could not be counted in this soil, nor could it be re-isolated either by enrichment of the soil with cellulose or inoculation of the soil on to a cellulose medium. It was presumed, therefore, that the organism had been killed out.

A similar pattern of events was followed in inoculated partially-sterilized Woburn soil (Expt. 4). There were six wide fluctuations in 32 days before the count steadied at $1\cdot0\times10^7$/g. soil. Following inoculation the count rose from $6\cdot0\times10^6$/g. to a maximum of $4\cdot7\times10^8$/g. soil. A count was made at 76 and 107 days of the total bacterial population (as appearing on yeast agar) in partially sterilized, uninoculated Rothamsted soil (Expt. 1). This population was greater ($9\cdot5\times10^7$/g. and $4\cdot9\times10^7$/g., respectively) than that in the similarly treated inoculated soil ($2\cdot1\times10^7$/g. and $3\cdot0\times10^7$/g., respectively). Thus, *Nocardia cellulans* appeared to have a depressing effect on the numbers of other bacteria. No antagonistic reaction between *N. cellulans* and the other bacteria was noted on the plates.

These experiments showed that *Nocardia cellulans* could be established in two different soil types, provided that the soils were partially or totally sterilized. The organism could not compete successfully with the microbial population in an untreated soil, and after a few weeks it disappeared.

Life cycle of Nocardia cellulans *in relation to the fluctuating numbers in the soil*

From previous observations on the morphology of *Nocardia cellulans* on different nutrient media (Metcalfe & Brown, 1957) it is known that the organism passes through a regular life cycle, from rod to mycelium to rod, remaining in this last form until re-inoculated to fresh media. It seemed possible that the marked fluctuations in numbers of *N. cellulans* in the soil, especially during the first month, might be correlated with this cycle. To see whether the cycle actually took place in the mass of the soil, morphological observations were made and the soil-shaking technique developed by Skinner (1951) used.

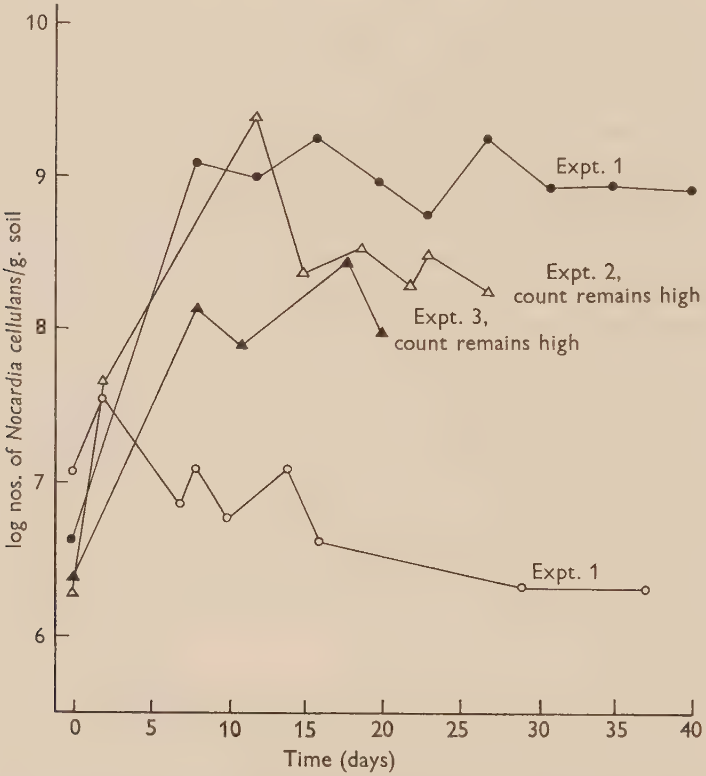

Fig. 1. Changes in the numbers of *Nocardia cellulans* inoculated into partially-sterilized soil and untreated Rothamsted soil. Exp. 1: ●, partially-sterilized soil; ○, untreated soil. Expt. 2: △, partially-sterilized soil. Expt. 3: ▲, partially-sterilized soil.

Preparation of material for morphological observations. Square 1 in. coverslips were coated on one side with plain 2 % (w/v) agar and drops of a tap-water suspension of a 24 hr. *Nocardia cellulans* culture put at the centres of the coverslips, which were then placed agar side down on to the surface of partially-sterilized and untreated Rothamsted soil (Expt. 5). Slides were prepared and treated in a similar way. A plate of plain agar was inoculated as control. The morphological changes in *N. cellulans* were observed daily by removing a slide and coverslip from the soil and examining these with a phase-contrast microscope. Slides were then replaced and re-examined the following day. The agar plate was also examined daily.

Examination of the coverslips in contact with the soil showed that within one day, on both treated and untreated soils, *Nocardia cellulans* had produced long mycelial threads radiating from the inoculation point. These threads began to segment, and by 5 days were divided along their length into short rods. By 7 days, new mycelial threads radiating towards the edges of the coverslips were produced from the tips of the old divided mycelia; these new threads then segmented. This process was repeated twice, by which time the threads had reached the edges of the coverslips and no further mycelium was produced. A summary of the changes in morphology during 26 days of observation is given in Table 1, since this shows that *N. cellulans* went through 4 complete cycles on both types of soils. These cycles were completed in 21 days on partially sterilized soil and in 17 days on untreated soil.

Table 1. *Changes in morphology of* Nocardia cellulans *during* 26 *days of observations made on inoculated coverslips placed in contact with partially sterilized and untreated soils.*

Day		Partially-sterilized soil	Untreated soil	
1	1	Germinating cells, long branching filaments	Same	1
	2	Filaments beginning to segment	Same	
	3	Segmentation continuing	Same	
	5	Segmentation to short rods complete	Same	
	6	Short rods	New unsegmented filaments produced from tips of divided mycelia	2
2	7	New unsegmented filaments produced from tips of divided mycelia	Segmentation of new filaments commencing	
	9	Segmentation of new filaments commencing	Segmentation complete. Short rods	
3	12	Segmentation complete. New mycelium at tips	New mycelium produced at tips	3
	13	—	Segmentation commencing	
	14	Segmentation commencing	Segmentation complete. Short rods	
	15	Segmentation complete. Short rods	New mycelium produced at tips, growing to edge of coverslip	4
	16	Short rods	Segmentation commencing	
4	17	New mycelium produced at tips, growing to edge of coverslip	Segmentation complete. Short rods	
	18	—	Short rods	
	19	Segmentation commencing	Short rods	
	20	Segmentation continuing	Short rods	
	21	Segmentation complete. Short rods	Short rods	
	22–26	Short rods	Short rods	

The repetitive cycle was also observed on the slides. There were 6 cycles in 23 days but, although the mycelium had not reached to the edges of the slides, the *Nocardia cellulans* remained in the rod form and stayed as such for a further

32 days, this being the whole time of observation. On plain agar, after the first mycelial production followed by segmentation there was no further morphological change.

The results of these observations indicated that cyclical development of *Nocardia cellulans* could take place when the organism was in surface contact with the soil and that these cycles occurred at the same time as the wide fluctuations in numbers of *N. cellulans* were occurring in partially-sterilized soil, that is, during the first 3–4 weeks following inoculation. Also, on untreated soil the completion of the cycles in 17 days corresponds to the period of wide fluctuations in the numbers of *N. cellulans* found after inoculation into such a soil, as found in Expt. 1. This, however, is not proof that cyclical development occurs in the mass of the soil, but evidence of such was obtained from shaking experiments.

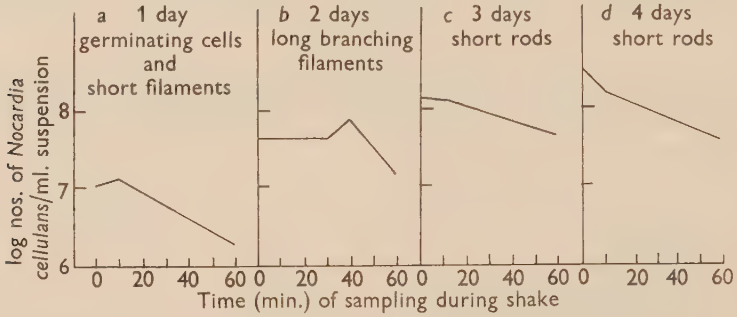

Fig. 2. Changes in numbers of *Nocardia cellulans* in a suspension of a pure culture shaken for 1 hr.

Technique for shaking soil samples. Skinner (1951) showed that when shaken in suspension, vegetative mycelium of *Streptomyces albidoflavus* broke up into a large number of viable fragments which were killed when shaking was prolonged; spores did not break up and were more resistant than vegetative fragments to shaking. He was able to detect very small quantities of mycelium and spores in a soil suspension by his technique. Shaking was performed by a reciprocating machine at 265 strokes/min. with a vertical movement of 9 cm. A preliminary experiment was performed with a pure culture of *Nocardia cellulans*, shakings being made daily for 4 days starting with a 24 hr. culture. A suspension (varying from 0·6 to $3{\cdot}5 \times 10^8$ cells/ml.) of each culture was made in 10 ml. sterile tap-water and added to 5 g. fine sand in a sterile 1 oz. screw-capped bottle, which was shaken for 1 hr. on the reciprocating machine. The numbers of *N. cellulans*/ml. suspension were estimated from 0·5 ml. samples taken at zero time before shaking began, and thereafter at 10 min. intervals. Twofold dilution series were prepared and the same technique used for estimations as for the soil counts. The counts were compared with visual observations of the morphology at the beginning of each period of shaking. The results obtained are shown in Fig. 2. With a 24 hr. culture of germinating organisms and short filaments the count rose slightly in 10 min. and then fell steadily

(Fig. 2*a*). A 2-day culture composed of long branching filaments gave a rise in count in 40 min. followed by a sharp fall (Fig. 2*b*); 3- and 4-day cultures, composed of short rods, showed a steady decline in count (Fig. 2*c*, *d*). Thus the shaking method could be used to distinguish between the rod and mycelial stages of *N. cellulans* and was used for this purpose in the main experiments.

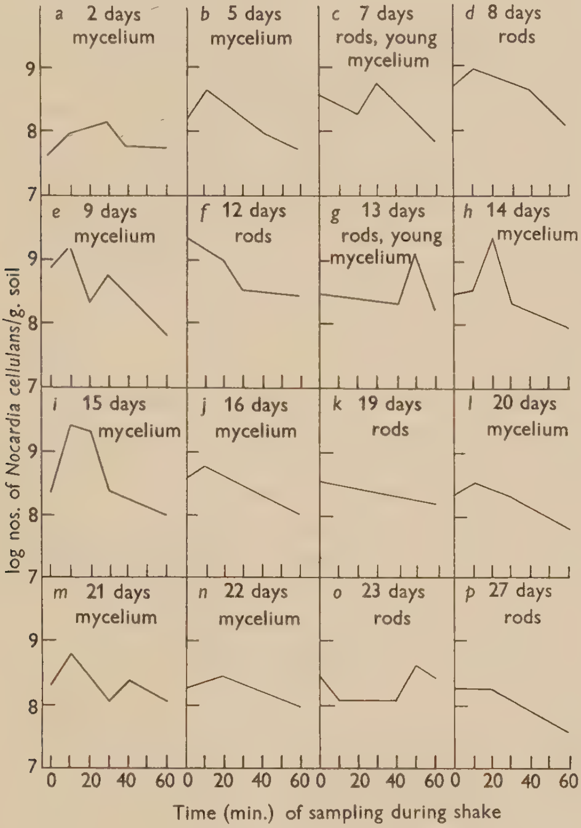

Fig. 3. Changes in the numbers of *Nocardia cellulans* in soil samples shaken for 1 hr. Soil from Expt. 2.

Two of the partially-sterilized Rothamsted soil samples, (Expts. 2 and 3), were examined by the shaking technique at intervals during 27 and 18 days respectively, the first examination being made immediately following inoculation. To do this small quantities of soil were taken from the flasks and suspended in 10 ml. sterile tap-water. This suspension was added to a bottle containing sand and sampled and shaken as before. The state of the *Nocardia cellulans* in the soil was deduced from the counts obtained.

The results obtained from shaking the sample at intervals for 27 days are shown in Fig. 3, but both experiments gave similar data. Following the observations with pure cultures an increase in the viable count with time of shaking, followed by a decrease was taken to indicate that *Nocardia cellulans* was predominantly mycelial (Fig. 3*a*), and a gradual decline, that rods were predominant (Fig. 3*f*, *k*). An interesting feature sometimes observed was a sudden increase in count which occurred at the 30, 40 or 50 min. time of sampling (Fig. 3*c*, *g*); this might be interpreted as a fragmentation of young mycelium which occurred only after prolonged shaking. On any one day when the initial counts indicated a predominance of rods and an increase in count occurred with continued shaking, the counts for the following day showed that the mycelial stage was again predominant (Fig. 3*c*, *d*, or 3*g*, *h*). Thus the initial stages of mycelial production could be detected.

This repetitive life cycle can be related to the fluctuations in *Nocardia cellulans* numbers in the soil. The graph shown in Fig. 1, Expt. 2, is the daily count of *N. cellulans* in the soil at zero time before shaking the sample. This should be studied in conjunction with the graphs in Fig. 3, which show the results of shaking this same sample. It is seen that a peak count corresponds to a predominance of rods (Fig. 3*f*, *k*, *o*). The soil count decreases during the development of mycelium and the count rises again as the mycelium fragments. During the first 12 days, when the soil count is rising to a maximum, the *N. cellulans* apparently passes through two life cycles. This same phenomenon was noted in the second experiment (the 18-day shake) where there was a peak which occurred at 8 days associated with one life cycle, and a maximum peak at 18 days associated with two life cycles (Fig. 1*c*, Expt. 3). After the wide fluctuations which occurred during the first month the count became steadier and the *N. cellulans* remained in the rod form. A shaking experiment performed on a soil sample in which *N. cellulans* was well established, 315 days after inoculation, indicated that rods were predominant. It is thought that actinomycetes and fungi exist in the soil most of the time as rods or spores; *N. cellulans* is probably no exception and once established it remains as a rod. The behaviour of *N. cellulans* when inoculated into partially sterilized soils alien to that from which it was isolated would suggest the possibility of other organisms, such as streptomycetes, becoming established for an indefinite period in partially sterilized but alien soil. The establishment of such an organism selected for its antagonistic action towards plant pathogens would be a step towards an effective means of controlling some plant diseases.

I wish to thank Dr H. G. Thornton, For.Sec. R.S., for his interest in the work and for criticizing the manuscript, Miss Jill Royston for her technical assistance and the Statistics Department, Rothamsted, for their advice.

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An Investigation of Fungistasis in Nigerian Soils

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SUMMARY: The presence of a fungistatic factor in Nigerian soils was demonstrated by the use of a technique in which disks of agar were placed on filter-paper in contact with moist unsterile soil and inoculated with fungal spores. After incubation the disks were examined and the percentage of germinated spores recorded. Inhibition of spore germination was not caused by autoclaved soil, nor when 0·5 % (w/v) glucose was present in the agar. The presence or absence and the type of vegetation had little effect on the fungistatic power of the soil. In two profiles examined, the property of fungistasis was confined, approximately, to the upper 40 cm. of the soil. Of nineteen species of fungi tested by the agar disk method, the germination of eleven was significantly inhibited, of seven unaffected and of one stimulated. The data obtained suggest that the phenomenon described in Nigerian soils is similar to that reported from other parts of the world.

Several workers have reported the occurrence of factors toxic or inhibitory to the growth of soil fungi and bacteria in a variety of natural soils in different countries. In Britain, Dobbs & Hinson (1953) reported complete inhibition of spore germination of *Penicillium frequentans* by the following soils, which came from different localities in England and Wales: five forest soils, five grassland soils including acid and calcareous types, three garden soils of differing fertility levels and three waterlogged soils. The work of Neilson-Jones (1941), Brian, Hemming & McGowan (1945) and Jefferys & Hemming (1953) was concerned with an acid heath soil from Wareham Heath in Dorset, which supported poor tree growth and was found to be toxic to fungi and bacteria. Neilson-Jones also detected some toxicity in 'normal' heath soil from Oxshott in Surrey. Hessayon (1953) found a factor inhibitory to *Trichothecium roseum* to be present in larger quantities in a clay than in a sandy soil. Park (1955, 1956) used a light garden loam in his studies in which he observed the lysis of fungal spores in contact with this soil. Winter (1940, 1955), working in Germany, obtained soil extracts inhibitory to *Ophiobolus graminis*, and extracts of pine litter and lysimeter water which were both shown to contain antibiotic substances. The leaf litter of several different tree species in Sweden has been found to exert an antibiotic effect on mycorrhizal fungi (Melin, 1946); while in France, Pochon & Barjac (1952) demonstrated that aqueous extracts of peat soils have a strong inhibitory effect on some bacteria, in particular *Azotobacter chroococcum*.

One of the earliest reports of toxic material in soils is that of Greig-Smith (1911), who found that Australian soils would yield aqueous extracts toxic to *Bacillus prodigiosus* (*Serratia marcescens*) and to a lesser extent to other bacteria. In America evidence has been obtained for the presence of inhibitory material in subsurface soils (Newman & Norman, 1943) and Canadian workers (Simmonds, Sallans & Ledingham, 1950; Chinn, 1953) have detected fungistatic activity towards *Helminthosporium sativum* and some other fungi in several soils tested. Stover (1955), in a study of *Fusarium oxysporum* f. *cubense* in Honduras, observed that the spores of this fungus germinated poorly or not at all in unsterile soil, unless glucose, plant sap or other nutrients were added; this appears to be the only report of the inhibitory action of soil from the tropics.

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The present work was undertaken to determine whether or not soil fungistasis, similar to that reported from other countries, occurs in the southern part of Nigeria, a region which lies within the Humid Tropics and has an annual mean temperature between 75° and 85° F. (*c.* 20–30° C.) and an annual rainfall of 40–80 in. When fungistasis was observed, some of the aspects of its distribution and properties were studied.

METHODS

Organisms. The following fungi were used: *Mucor fragilis* Bain., Commonwealth Mycological Institute (IMI) 54600; *Cunninghamella elegans* Lendner, IMI 54404; *Ustilago zeae* (Beckm.) Unger; *Puccinia polysora* Underw.; *Aspergillus niger* van Tiegh., IMI 54399; *Aspergillus violaceo-fuscus* Gasp., IMI 54401; *Aspergillus fischeri* Wehm., IMI 54403; *Penicillium citrinum* Thom, IMI 54218; *Penicillium terrestre* Jensen series, IMI 54216; *Penicillium verruculosum* Peyronel, IMI 54220; *Penicillium avellaneum* Thom & Turesson, IMI 54225; *Paecilomyces varioti* Bain., IMI 54401; *Gliocladium deliquescens* Sopp series, IMI 54219; *Myrothecium verrucaria* Ditmar ex Fries, IMI 54694; *Fusarium solani* sensu Snyder & Hansen, IMI 54696; *Fusarium equiseti* (Corda) Sacc., IMI 54697; *Cladosporium cladosporioides* (Fres.) de Vries, IMI 54705; *Helminthosporium ravenellii* Curt.

These fungi, with the exceptions of *Ustilago zeae*, *Puccinia polysora* and *Helminthosporium ravenellii*, were all isolated from soil at Ibadan, and were identified or their identities confirmed by the Commonwealth Mycological Institute, whose acquisition numbers are given. The material of the plant pathogenic fungi was freshly collected at Ibadan.

Media. Distilled water agar (2·0 %): 2·0 % (w/v) Difco 'Bacto-Agar' in glass-distilled water, autoclaved at 120° for 20 min. Czapek agar: $NaNO_3$, 2·0 g.; K_2HPO_4, 1·0 g.; $MgSO_4.7H_2O$, 0·5 g.; KCl, 0·5 g.; $FeSO_4$, 0·1 g.; sucrose 30·0 g.; agar 15·0 g.; tap water to 1000·0 ml.; autoclaved at 120° for 20 min. Lactophenol (Amann): phenol, 20 g.; lactic acid, 20 g.; glycerol, 40 g.; water, 20 g.

The agar disk method for assessing soil fungistasis

When a study of soil fungistasis was started, it was thought desirable to devise a simple method for assessing the fungistatic activity of soils or for comparing the sensitivity of different fungi to fungistasis, which would yield quantitative data suitable for statistical analysis. Techniques for detecting the presence of inhibitory factors in soil dependent upon diffusion from soil into agar have been used by Neilson-Jones (1941), Hessayon (1953), Jefferys & Hemming (1953) and Witkamp & Starkey (1956), while Dobbs & Hinson (1953) detected fungistatic material diffusing from soil through cellulose film. The buried-slide technique of Chinn (1953), in which a glass slide coated with a spore suspension in agar is buried in the soil and later recovered for examination, can yield qualitative and quantitative data on the effects of inhibitory soil factors on spores. This technique provides a rather more sensitive test for fungistasis than the agar disk method to be described, but was found less convenient to handle and the results more difficult to record quantitatively in routine tests. For special studies (Jackson, 1957) Chinn's technique has been found invaluable. Preliminary experiments, in which cellulose film squares were placed in contact with the surface of moist soil and inoculated on the upper surface with fungal spores, showed that although such squares could be readily removed to a microscope slide for examination, unsatisfactory results were obtained owing to uneven contact between the soil and the film and also to partial drying of the cellulose. As an alternative to cellulose film, the possibility of using agar disks placed in contact with the soil was examined and the following method developed.

Preparation of soil samples. Composite samples were passed through a 2 mm. sieve, either immediately after collecting or after air-drying for 12 hr., and thoroughly mixed before testing. Forty to 60 g. of soil were placed in a clean 85 mm. Petri dish and brought to approximately 60 % of water-holding capacity by the addition of distilled water.

Agar disks. These were prepared by pouring 9 ml. of melted 2 % (w/v) distilled-water agar into a selected flat-bottomed 85 mm. Petri dish standing on a level surface, giving a layer of agar just under 1·5 mm. in thickness. The disks were cut from the agar with a flamed 7·5 mm. diameter cork-borer and removed on the tip of a flamed scalpel.

Test organism. The organism used was a strain of *Penicillium citrinum*, isolated from soil at Ibadan, and was used in all experiments with the exception of those in which the sensitivities of different fungi to soil fungistasis were being examined. *P. citrinum* was chosen on account of the initial high percentage germination of its conidia on water agar, its sensitivity to soil fungistasis and the rapidity with which it produced heavy crops of spores on nutrient media. Spore suspensions were prepared from young, heavily sporulating cultures on Czapek agar.

Procedure. In the first tests the agar disks were placed in direct contact with the soil. It was found that three disadvantages were associated with this procedure, namely, uneven contact between the agar and the soil, adhesion of soil particles to the agar on removing the disk from the soil and the growth of fungal hyphae from the soil into the agar. The first of these difficulties was decreased and the second and third of them eliminated by interposing one thickness of Whatman's no. 1 filter-paper between agar disk and soil, with only a slight decrease in the sensitivity of the test. For each test, four 1 cm. squares of filter-paper are placed on the surface of the soil in a Petri dish and an agar disk placed on each of the squares. Either immediately, or after 1–4 hr. of incubation to allow diffusion of inhibitory substances from soil into agar disk, the surface of each disk was inoculated with a drop of a spore suspension in distilled water on the end of a glass rod. Provided that control and treated disks were inoculated with samples of the same spore suspension, standardization of the suspension was not necessary. Controls were similarly prepared, but the agar disks were placed on filter-papers saturated with distilled water in dishes containing no soil. After inoculation, the Petri dish with lid replaced was incubated at room temperature (20–27° in Ibadan) or at 25° in an incubator for the minimum period required to obtain good germination of the spores on the control disks, which was 16 hr. with *Penicillium citrinum*.

After incubation the agar disks were removed to microscope slides and covered with a drop of lactophenol and a coverslip. Germinated and ungerminated spores in each of four random microscope fields on each agar disk were counted, the magnification used depending on the size and density of distribution of the spores. Spores were recorded as having germinated when they had produced a germ tube of one spore diameter or more in length. Results are expressed as the % of the total number of spores that had germinated, and, after angular transformation, may be submitted to analysis by Student's *t* test or analysis of variance.

RESULTS

Demonstration of soil fungistasis and the effect on this of autoclaving the soil

The first soil sample examined for inhibition was a valley slope soil (pH 6·4) from the University College site, Ibadan, which had for the 3 years before sampling carried a stand of young teak trees (*Tectona grandis*). The sample was a composite one collected from the upper 8 cm. of soil and air-dried before bringing to 60 % water-holding capacity and testing (air-drying for short periods was found to have no appreciable effect on soil fungistasis). After moistening, one part of the sample was tested without further treatment, while another part was autoclaved at 120° for 30 min. before testing. The agar disk method was used with *Penicillium citrinum* as the test organism. The results of the spore germination counts from this experiment are presented in Table 1, together with the analysis of variance table. The results of further experiments in which the agar disk method was used are expressed as mean % and mean transformed % germinations, with an indication of the significance of the means. While the untreated soils tested in this experiment almost completely inhibited germination of the test organism, autoclaving removed this effect and resulted in a degree of germination not significantly different from that of the control.

The effect of glucose on the inhibitory factor

Dobbs & Hinson (1953) reported that as little as 0·1 % (w/v) glucose + inorganic salts added to their soil resulted in the disappearance of the fungistatic effect. In the present work a test was carried out at the same time and with part of the same soil sample as was used in the previous experiment, to determine the effect of adding glucose to the agar used in preparing the agar disks. While the concentration of glucose was likely to remain substantially constant in the agar disks during a test, a small concentration of glucose added to unsterile soil might be expected to start decreasing appreciably within a few hours of addition. In this experiment 0·5 % (w/v) glucose was added to the agar in the treated and control disks, and *Penicillium citrinum* was used as the test organism. It was found that no significant inhibition of spore germination occurred on agar disks containing 0·5 % (w/v) glucose placed over the soil.

Table 1. *The germination of* Penicillium citrinum *conidia on agar disks over filter-paper, untreated soil and autoclaved soil*

		Filter-paper (control)				Untreated soil				Autoclaved soil			
		Disk number											
		1	2	3	4	1	2	3	4	1	2	3	4
Field		Count (germinated and ungerminated conidia)/4 microscope fields											
1	Germinated	68	98	93	98	2	1	0	3	32	88	54	71
	Ungerminated	17	21	13	35	116	101	53	225	7	31	9	47
2	Germinated	50	77	63	82	3	0	0	1	62	99	62	98
	Ungerminated	15	20	4	16	59	94	46	112	5	23	10	39
3	Germinated	55	68	76	90	2	1	0	1	98	59	61	94
	Ungerminated	9	8	14	25	80	21	143	103	10	14	22	47
4	Germinated	61	59	114	80	3	0	0	1	67	75	61	72
	Ungerminated	11	12	22	15	93	71	125	131	10	22	17	37
	% germinated	81·8	83·2	86·7	79·4	2·8	0·7	0	1·0	89·0	78·1	80·4	66·3
		Mean 82·8				Mean 1·1				Mean 78·4			
	Angular transformation	64·9	65·6	68·9	62·7	10·0	5·7	0	5·7	70·6	62·0	63·4	54·3
		Mean 65·5*				Mean 5·3*				Mean 62·6*			

Analysis of variance

Source of variance	Sums of squares	Degrees of freedom	Mean squares
Between columns	9205·9	2	4602·9
Within columns	204·2	9	22·7

* Significant difference between means for $P=0{\cdot}05$ is 7·7.

Fungistatic nature of the inhibition

The very poor germination of *Penicillium citrinum* spores over untreated soil after 16 hr. as compared with excellent germination in the absence of soil might have been due either to a slowing in the rate of germination or to complete prevention of the process in most of the spores. In the latter case the spores might have lost their capacity for germination and have become non-viable, or have retained viability, being able to germinate when removed from the influence of the soil or affected by a stimulatory substance. The possibility that the soil delayed rather than prevented germination was examined by setting up an experiment with the same soil and organism as used in the previous tests, but using sufficient agar disks to allow for the removal of four every 24 hr. over a period of 1 week, so that any increase in germination during that period could be observed. Observations were not recorded after the fifth day as the growth of fungal and bacterial contaminants on the agar disks made further accurate counts impossible. The results recorded in Table 2 show that by the end of 5 days no significant increase in % germination had occurred. It was observed in this experiment, as in others, that the small % of spores which did germinate in the presence of the soil factor developed at a slower rate than in the absence of soil.

Table 2. *The effect on the germination of* Penicillium citrinum *conidia of prolonging incubation over soil*

Duration of incubation (hr.)	Mean germination (%)	Mean of transformed % germination
16	0·8	4·9
40	2·4	7·0
64	7·7	13·2
88	1·3	4·9
112	4·2	10·4

Differences between means not significant at $P = 0{\cdot}05$.

To determine whether or not the inhibitory soil factor is toxic, four inoculated disks which had been in contact with soil for 16 hr. were removed for microscopic examination and subsequently placed on filter-paper moistened with 1·0 % (w/v) solution of glucose in distilled water and incubated for a further 24 hr. before re-examination. The % germination of the spores on these disks reached a value near that of those on the control disks which had not been in contact with the soil. The results of this experiment clearly demonstrate that, at least for *Penicillium citrinum* and with a limited period of exposure under the conditions of the experiment, the soil inhibitory factor had no permanent effect on the germinative capacity of the spores and may therefore be defined as a fungistatic rather than fungicidal.

Table 3. *The effect on the germination of* Penicillium citrinum *conidia of transferring inoculated disks from soil to glucose-moistened filter-paper*

Treatment	Mean germination (%)	Mean of transformed % germination
16 hr. over soil	1·7	7·4*
16 hr. over soil + 24 hr. over filter-paper	70·3	57·2
16 hr. over filter-paper	72·5	58·4

* Significant difference between means for $P = 0{\cdot}05$ is 26·2.

The occurrence of inhibition of fungal growth in different Nigerian soils

In addition to samples from the University College site at Ibadan in the Western Region of Nigeria, some soils from other parts of Nigeria were examined for fungistasis. Samples were collected from the following localities:

(1 and 2) Between Ilorin and Bode Sabu, Ilorin Province, Northern Region; well-developed Guinea Savannah (*Encephalartos barteri* belt), uncultivated for a considerable period, mixed grass and scrub vegetation. Annual rainfall *c.* 49 in.

(3 and 4) Between Oyo and Iseyin, Oyo Province, Western Region; derived Guinea Savannah, probably cultivated within the last 4 years but at the time of sampling under mixed grass fallow. Annual rainfall *c.* 46 in.

(5 and 6) Asijere, Oyo Province, Western Region; mixed secondary rain forest, estimated to be more than 20 years old. Annual rainfall *c.* 48 in.

(7) Lagos, Federal Region; mangrove (*Rhizophora racemosa*) swamp. Annual rainfall *c.* 72 in.

(8) Between Lagos and Ikorodu, Federal Region, freshwater swamp with mixed vegetation. Annual rainfall *c.* 72 in.

The samples were all composite ones collected from the upper 10 cm. of the soil. Soil samples nos. 1–6 inclusive may be broadly described as moderately to strongly leached porous sandy clays, low in organic matter except for the forest soil samples (5 and 6) which contained a considerable quantity of organic matter in the upper 10 cm. Sample no. 7 from a mangrove swamp was a silty clay containing a mass of *Rhizophora* rootlets, while sample no. 8 from a freshwater swamp was a sandy soil rather rich in organic matter. No pH measurements were made on these samples. The indicated rainfall figures are necessarily approximate as records are available only from a limited number of stations.

The samples were tested for inhibition by the agar disk method with *Penicillium citrinum* as test organism. Samples nos. 1–6 inclusive were tested together with one control, while samples 7 and 8 were tested at a later date with a different control. As shown in Table 4, significant inhibition was caused by all the soils tested, although the freshwater swamp soil, which was waterlogged at the time of sampling, produced a smaller effect than the other soils.

Table 4. *The effect of different soils on the germination of* Penicillium citrinum *conidia*

Soil sample no.	Mean germination (%)	Mean of transformed % germination
1	3·5	10·7*
2	4·6	12·2
3	1·4	3·4
4	8·4	16·5
5	6·1	14·1
6	13·0	20·0
Filter-paper control	60·3	51·1

* Significant difference between means for $P=0{\cdot}05$ is 7·2.

Soil sample no.	Mean germination (%)	Mean of transformed % germination
7	0	0
8	59·2	50·3*
Filter-paper control	85·8	67·8

* Significantly different from the control at $P=0{\cdot}01$.

The effect of vegetation on soil fungistasis

The effect of vegetation on the inhibitory activity of similar soils was studied in a series of plots receiving different treatments. The plots were established on an area of the University College farm which had been under secondary bush vegetation for at least the last 20 years, during which period it had not been cultivated. The area was cleared of vegetation in October 1952, and laid down to the following plots, each 6 × 90 ft., in August 1953 (during the interval between clearing and laying down the plots the soil was rapidly covered by a ground layer of herbaceous weeds): (1) Bare fallow—regularly weeded to keep the plot as nearly as possible free from vegetation. (2) Regenerating vegetation—plot untreated; natural vegetation allowed to regenerate. (3) Planted with Giant Star Grass (*Cynodon plectostachyum*). (4) Planted with the leguminous cover-crop, Tropical Kudzu (*Pueraria phaseoloides*). Composite soil samples were taken from the upper 8 cm. of each plot in July 1955, by which time plots 2–4 were thickly covered by vegetation below which was a well-developed layer of organic litter. In addition to the samples from the above plots, one composite sample from another part of the farm, carrying a crop of maize, was included in the test for comparative purposes. The agar disk method was again used with the same test organism.

The results (Table 5) of this experiment indicate that all the soil samples caused a highly significant inhibition of spore germination as compared with the control, the inhibition caused by soil from plots nos. 1 and 3, and the maize plot being significantly greater than that produced by soil from plot 2, carrying regenerating vegetation. The soil from plot 1, which had been exposed to the full effects of leaching and insolation for nearly 2 years, showed the highest degree of inhibition.

Table 5. *The germination of* Penicillium citrinum *conidia over soils which have carried different types of vegetation*

Plot no.	pH value	Mean germination (%)	Mean of transformed % germination
1	6·2	0·3	0·7
2	6·6	4·3	11·7
3	6·4	1·5	3·5
4	6·3	1·6	4·9
Maize plot	6·0	0·4	2·1
Filter-paper control	—	76·8	61·5

* Significant difference between means for $P = 0·05$ is 7·2.

The vertical distribution of soil fungistasis

The distribution of inhibition in two soil profiles was examined. Profile 1 was a hill-top series soil and carried a mixed secondary vegetation with a well-developed herb and scrub layer, while profile 2 was a valley slope soil with a thin grass cover of *Sporobolus pyramidalis* and *Panicum maximum*; both soils were on the University College site. Profile 1 was sampled from the exposed face of a freshly dug pit at intervals to a depth of 183 cm., the samples from the second profile being collected with a screw auger to a depth of 91 cm. The samples were tested by the agar disk method with *Penicillium citrinum* as the test organism. The results are represented graphically in Fig. 1. In both the profiles inhibitory material was present in the samples taken down to 30 cm.

but not in the next lower samples, which showed a slight but significant, stimulation, which was not present at 91 cm. in the second profile. The pH values of the samples from profile 1 ranged from 5·6 to 6·0 and of the samples from profile 2 from 5·6 to 6·6.

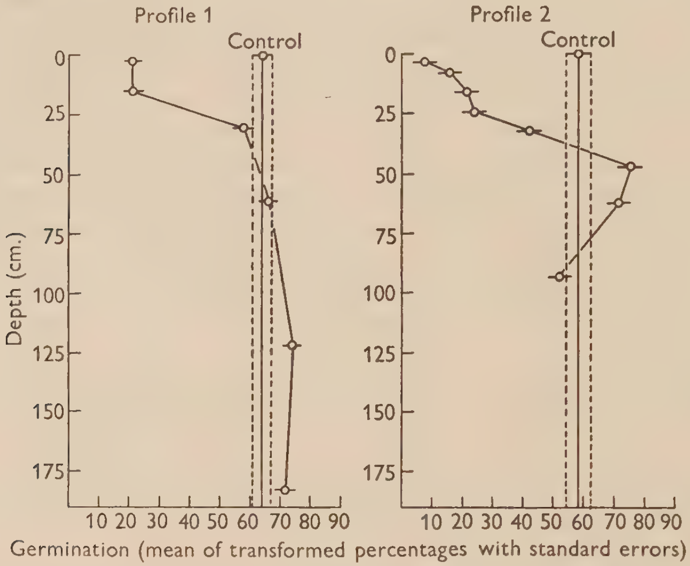

Fig. 1. The effect of soil samples taken from different depths in two profiles on the germination of *Penicillium citrinum* conidia. Germination in the absence of soil is indicated by a vertical line on each graph.

The sensitivity of different fungi to soil fungistasis

Preliminary results obtained while a suitable test organism for use in the agar disk test was being sought had indicated that there existed considerable differences between the sensitivities of different fungi to the inhibitory effect of soil. Sixteen fungi isolated from local soils and three plant pathogenic fungi were tested for sensitivity to Ibadan soil. All the fungi were tested by the agar disk method against a sample of the same soil as used in the first experiment described, a reference disk inoculated with *Penicillium citrinum* being included in each soil plate. Conidial suspensions were obtained from Czapek agar-plate cultures incubated for a sufficient period to allow of good sporulation, except in the case of *Ustilago zeae*, *Puccinia polysora* and *Helminthosporium ravenellii*, where brandspores, uredospores and conidia, respectively, were obtained directly from infected plant material. The fungi tested gave results which allow them to be placed in three categories according to whether germination of their spores was significantly (at $P = 0{\cdot}05$) inhibited, unaffected, or stimulated, respectively, by soil as compared with spores incubated over moist filter-paper. Inhibited were: *Mucor fragilis*, *Ustilago zeae*, *Penicillium citrinum*, *P. terrestre*, *P. verruculosum*, *Aspergillus niger*, *A. violaceo-fuscus*, *Gliocladium deliquescens*, *Trichoderma viride*, *Cladosporium cladosporioides*, *Myrothecium verrucaria*; unaffected were: *Cunninghamella elegans*, *Puccinia polysora, Penicillium avellaneum, Aspergillus fischeri, Paecilomyces varioti, Fusarium solani, Fusarium equiseti*; stimulated was: *Helminthosporium ravenellii*. While a significant inhibition on agar disks over soil may be taken as indicative of a strong inhibition in the soil, lack of inhibition on agar disks cannot be taken as proof of no inhibition in the soil.

DISCUSSION

The results obtained in the experiments described indicate the common occurrence in soils of south-western Nigeria of inhibitory material effective against certain fungi and similar to (or identical with) that described by workers in other countries. This similarity is suggested by the following features of the inhibition studied in Nigeria: (1) the presence in all soils tested of water-diffusible material which inhibits the germination of the spores of several different species of fungi; (2) the fungistatic properties of this material; (3) disappearance of the inhibition on autoclaving the soil; (4) counteraction of the effect by glucose. Although it was possible to examine only a limited number of different soils, the fact that all the soils tested showed some degree of fungistatic activity would suggest that the majority of Nigerian soils might be expected to show a similar effect. The rather low activity in a waterlogged, freshwater swamp soil might be accounted for by decreased production of the inhibitory material under partially anaerobic conditions or by some loss of the factor resulting from prolonged leaching.

An interesting feature of the plot experiment was the presence of a strong fungistatic effect in soil from the bare fallow plot after nearly 2 years of subjection to repeated intense insolation and leaching. During this period no additions of organic or inorganic material were made to the plot, so that latterly the activity of micro-organisms might have been expected to have been low. It would appear from these considerations that, if the material is microbial in origin, it must possess a high degree of resistance to leaching and to chemical and biological inactivation in the soil. Inhibition has been reported in surface and subsurface soils down to a depth of 4 ft. (Newman & Norman, 1943; Dobbs & Hinson, 1953), but in the present study was not found below 30 cm. (1 ft.) in the two profiles examined. The greatest inhibitory effect was found to correspond approximately with the zone in which the greatest concentration of roots was present.

Of the fungi tested for sensitivity to fungistasis by these soils, *Aspergillus niger* and *Trichoderma viride* represented the two species most commonly isolated from soil at Ibadan, either by the dilution plate or by Warcup's soil-plate method (Warcup, 1950). The other fungi whose spores were inhibited were also frequently isolated from local soils, with the exception of the plant pathogen *Ustilago zeae*. The sensitivity of the brandspores of this fungus to soil fungistasis may be associated with the fact that under natural conditions they frequently reach the soil before germination.

The confirmation by the present work of the occurrence in tropical soils of material which inhibits the germination of fungal spores, considered together with reports of a similar phenomenon in geographically widely separated areas, strongly suggests that such material may be almost universally present in soils. There can be no doubt that the existence of inhibitory substances in soils, the effects of which are only overcome under specific conditions, must exert a profound effect on the biology of soil-inhabiting fungi.

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Criteria for the Growth of Contaminants and Mutants in Continuous Culture

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SUMMARY: Criteria are given for the establishment of growth of contaminants and mutants in continuous cultures of the Monod type. These explain, in particular, why contaminants so rarely interfere with the operation of such cultures.

In this paper I investigate the conditions under which the population of bacteria in a continuous culture of the Monod (1950) type may be displaced by foreign organisms, either mutants or contaminants. I have not attempted a full discussion of the balance between mutants and parent organisms, my object being mainly to analyse the danger of infection which besets the working of preparative cultures. The paper falls into two parts. The first concerns what may be called the biochemical criteria for successful displacement; in it the organisms are treated as chemical reagents, sensibly devoid of structure, and certain necessary conditions follow. The second concerns the statistical criteria which give the probabilities for successful displacement. I adopt the nomenclature and symbols of Herbert, Elsworth & Telling (1956), in which the fundamental differential equations for conditions in the culture vessel are

$$\frac{dx}{dt}=x\left(\mu_m\frac{s}{K+s}-D\right), \tag{1a}$$

$$\frac{ds}{dt}=(s_R-s)\,D-\frac{x}{Y}\,\mu_m\,\frac{s}{K+s}, \tag{1b}$$

where t = time,
x = concentration of organisms by mass,
s = concentration of limiting substrate,
μ_m = maximum mass growth-rate constant for the organism and medium concerned,
Y = yield constant for the given organism and substrate,
K = saturation constant for the given organism and substrate,
D = dilution rate,
s_R = concentration of limiting substrate in ingoing medium.

I assume throughout (i) that D is held at a constant value, (ii) that before the entry of foreign organisms the culture has had time to reach a steady state, (iii) that the growth rate is limited by deficiency of a single nutrilite, (iv) that there is no interaction between two kinds of organisms but their competition for that nutrilite, (v) that growth rates adjust themselves to changes in substrate concentration without appreciable lag, and (vi) that the mixing in the culture vessel is 'perfect', i.e. that the ingoing liquid is instantaneously and uniformly dispersed throughout the working volume. These restrictions simplify the analysis; they can be relaxed to some extent, but I do not think that a more general discussion would be of greater value in the present state of knowledge.

In many simple examples it is found that over a wide range of substrate concentration (s) the growth-rate constant (μ) of the organism depends only on s. The form

$$\mu(s) = \mu_m \frac{s}{K+s}, \tag{2}$$

chosen in (1a), (1b) then appears to represent the relation between μ and s with sufficient accuracy for practical purposes. (K is usually very small. Spicer (1955), takes $\mu \propto s$. This is approximately true in continuous cultures only when D is not near its critical value

$$D_{\text{cr}} = \mu_m \frac{s_R}{K+s_R},$$

unless s_R is itself much less than K.) When a culture has been running for a long time (compared with $1/D$) we have

$$s + x/Y = s_R,$$

i.e. the substrate concentration plus the mass concentration of organisms reckoned as substrate must equal the substrate concentration in the inflowing medium, whatever the initial values of x, s. We thus have ultimately

$$\mu = \mu_m \frac{s_R - x/Y}{K + s_R - x/Y},$$

and since x must be positive $d\mu/dx$ is negative. Hence (Spicer, 1955) there is a final steady state with $x = \tilde{x}$, $s = \tilde{s}$, $dx/dt = 0$ and

$$\tilde{s} + \tilde{x}/Y = s_R, \tag{3}$$

$$\mu = \mu_m \frac{\tilde{s}}{K+\tilde{s}} = D \quad (D < D_{\text{cr}}). \tag{4}$$

$$(\text{For } D \geqslant D_{\text{cr}},\ \tilde{x} = 0,\ \tilde{s} = s_R.)$$

It can also be shown that x and s have each at most a single maximum or minimum for finite t, whatever the initial conditions.

CHEMICAL KINETICS OF DISPLACEMENT

Suppose the culture to be running at a steady rate with $x = \tilde{x}$, $s = \tilde{s}$. Let a foreign kind of organism—a contaminant or mutant of a single variety—be introduced at a concentration y_0. Let its saturation constant be L and its maximum growth-rate constant λ_m. Then at the outset its concentration y will increase at a rate

$$\frac{dy}{dt} = y_0\left(\lambda_m \frac{\tilde{s}}{L+\tilde{s}} - D\right).$$

For this expression to be positive, we must have

$$\lambda_m \frac{\tilde{s}}{L+\tilde{s}} > D. \tag{5}$$

Eliminating $\tilde{s}$ between (4) and (5):

$$\frac{\lambda_m KD}{\mu_m L - DL + DK} > D.$$

Since we must have $D < \mu_m$ if $\tilde{x} > 0$, the denominator on the left is positive and

$$\lambda_m K - \mu_m L > D(K-L).$$

Hence, the concentration of the foreign organism can increase only if the following relation holds between λ_m, μ_m, K, L and D:

$$\left.\begin{aligned} \frac{\lambda_m K - \mu_m L}{K-L} &> D \quad (K > L), \\ \frac{\mu_m L - \lambda_m K}{L-K} &< D \quad (L > K). \end{aligned}\right\} \tag{6a}$$

Or, if D is eliminated from (4) and (5),

$$\left.\begin{aligned} \frac{\lambda_m K - \mu_m L}{\mu_m - \lambda_m} &> \tilde{s} \quad (\mu_m > \lambda_m), \\ \frac{\mu_m L - \lambda_m K}{\lambda_m - \mu_m} &< \tilde{s} \quad (\lambda_m > \mu_m). \end{aligned}\right\} \tag{6b}$$

The survival of the foreign variety thus depends not only on λ_m and L, but also on the level at which the culture is being run at the time of its appearance.

So far we have been concerned only with the first stages of the change in y. To discuss the final outcome when (5), (6a), (6b) hold, we need the following information:

(i) At $t=0-$, $x=\tilde{x}$. The appearance of a mutant at concentration y_0 implies that at $t=0+$, $x=\tilde{x}-y_0$, $=x_0$, say. If Z is the yield constant for the new variety, we then have

$$\begin{aligned} \tilde{s}+x_0/Y+y_0/Z &= \tilde{s}+x_0/Y+y_0/Y+y_0(1/Z-1/Y) \\ &= \tilde{s}+\tilde{x}Y+y_0(1/Z-1/Y) \\ &= s_R+y_0(1/Z-1/Y), \end{aligned}$$

and so if $Z > Y$
$$\tilde{s}+x_0/Y+y_0/Z < s_R, \tag{7}$$

and if $Z \leqslant Y$
$$\tilde{s}+x_0/Y+y_0/Z \geqslant s_R. \tag{8}$$

If instead y represents a contaminant—an addition to the population, not a replacement of part of it—$x_0=\tilde{x}$ and (8) holds in this case also.

(ii) At all subsequent times

$$\frac{dy}{dt} = y\left(\lambda_m \frac{s}{L+s} - D\right), \tag{9}$$

and (by an obvious extension of (1b))

$$\frac{ds}{dt} = (s_R - s)D - \frac{x}{Y}\mu_m \frac{s}{K+s} - \frac{y}{Z}\lambda_m \frac{s}{L+s}. \tag{10}$$

Multiplying (1a) by $1/Y$, (9) by $1/Z$ and adding to (10):

$$\frac{d}{dt}\left(s+\frac{x}{Y}+\frac{y}{Z}\right) = D\left\{s_R - \left(s+\frac{x}{Y}+\frac{y}{Z}\right)\right\},$$

whence
$$s+x/Y+y/Z=s_R+Qe^{-Dt}, \tag{11}$$
where the constant Q is to be determined by the initial values of s, x, y. If (7) holds, Q is negative; if (8) holds, it is zero or positive.

(iii) If x, y, s have limits as $t\to\infty$, say $\tilde{x}_1$, $\tilde{y}_1$, $\tilde{s}_1$, they must simultaneously satisfy
$$\tilde{x}_1\left(\mu_m\frac{\tilde{s}_1}{K+\tilde{s}_1}-D\right)=0, \tag{12a}$$
$$\tilde{y}_1\left(\lambda_m\frac{\tilde{s}_1}{L+\tilde{s}_1}-D\right)=0 \tag{12b}$$
(from (1a) and (9) by equating the derivatives to zero).

There are now two possibilities: (A) Equation (7) holds and Q is negative in (11); (B) Equation (8) holds and Q is zero or positive.

(A) At $t=0+$ by (5)
$$\frac{dy}{dt}>0, \tag{13}$$
and so if y does not approach a limit monotonically, the first extremum to occur must be a maximum. But for finite t
$$\frac{ds}{dt}+\frac{1}{Y}\frac{dx}{dt}+\frac{1}{Z}\frac{dy}{dt}>0$$
(from (11) with Q negative). At an extremum of y, $dy/dt=0$ and s has the value $\tilde{s}_1$ given by (12b). Comparison of (12b) with (5) shows that $\tilde{s}_1<s$, and $dx/dt<0$ (by (4)). Hence $ds/dt>0$. But
$$\frac{d^2y}{dt^2}=\frac{dy}{dt}\left(\lambda_m\frac{s}{L+s}-D\right)+y\lambda_m\frac{L}{(L+s)^2}\frac{ds}{dt}, \quad >0,$$
if $dy/dt=0$, $y>0$ and $ds/dt>0$; an extremum of y for finite t must be a minimum. Thus y has no extremum for finite t, and by (13) it must approach a limit $\tilde{y}_1>y_0>0$. Then $dy/dt\to 0$ and $s\to\tilde{s}_1$, $<\tilde{s}$; for large t
$$\frac{1}{x}\frac{dx}{dt}\to\left(\mu_m\frac{\tilde{s}_1}{K+\tilde{s}_1}-D\right), \quad <0;$$
dx/dt itself cannot remain finitely less than zero, since if it did, x would tend to $-\infty$. So $x\to 0$, and the new variety entirely supersedes the old.

(B) When Q in (11) is positive or zero
$$\frac{ds}{dt}+\frac{1}{Y}\frac{dx}{dt}+\frac{1}{Z}\frac{dy}{dt}\leqslant 0.$$
If x does not approach a limit monotonically, we have at every extremum (unless $x=0$)
$$s=\tilde{s} \quad\text{and}\quad dy/dt>0$$
by (4) and (5), and hence $ds/dt<0$. From (1a),
$$\frac{d^2x}{dt^2}=\frac{dx}{dt}\left(\mu_m\frac{s}{K+s}-D\right)+x\mu_m\frac{K}{(K+s)^2}\frac{ds}{dt}, \quad <0,$$
if $x>0$, $dx/dt=0$ and $ds/dt<0$; every extremum of x for finite t is a maximum. Now at $t=0+$, $s=\tilde{s}$ and $dx/dt=0$, so the origin is a maximum of x, and there can be no other; x approaches a limit $\tilde{x}_1<\tilde{x}$ monotonically. The limit must be

zero, for if it were not, we should have $s \to \tilde{s}$ and $dy/dt > 0$ again, and dy/dt cannot remain finitely greater than zero. Thus $dx/dt \leqslant 0$ and so $s \leqslant \tilde{s}$ for all t;

$$s + x/Y < \tilde{s} + \tilde{x}/Y = s_R.$$

Hence by (11) $$y > y_0.$$

Since y remains positive and $x \to 0$, we again reach a state where only one variety of organisms remains, and then continued oscillation is impossible.

Both (A) and (B) yield

$$y \to \tilde{y}_1 > 0,$$

$$x \to 0,$$

$$s \to \tilde{s}_1,$$

and (11) and (12*b*) show that

$$\tilde{s}_1 = LD/(\lambda_m - D),$$

$$\tilde{s}_1 + \tilde{y}_1/Z = s_R.$$

Analogous reasoning shows that if the sense of the inequalities (5), (6*a*), (6*b*) is reversed, the outcome is

$$y \to 0,$$

$$x \to \tilde{x},$$

$$s \to \tilde{s},$$

i.e. continued growth of the foreign variety cannot occur. However, a constant source of contamination by mutation or infection from without will give rise to a steady (but practically negligible) level of concentration of foreign organisms (Novick, 1955; Maxon, 1955), even though none is individually capable of sustained reproduction in the culture.

Finally if precisely

$$\lambda_m \frac{\tilde{s}}{L + \tilde{s}} = \mu_m \frac{\tilde{s}}{K + \tilde{s}} = D,$$

the two varieties can coexist indefinitely in any ratio of concentration; the outcome is

$$y \to \tilde{y}_1 > 0,$$

$$x \to \tilde{x}_1 > 0,$$

$$s \to \tilde{s}_1 = \tilde{s},$$

but $\tilde{x}_1$, $\tilde{y}_1$ will depend on the initial values x_0, y_0. If the foreign variety is a contaminant, or if it is a mutant with $\lambda_m \neq \mu_m$, $L \neq K$, the probability of the occurrence of this condition is infinitesimal. In practice coexistence could only be maintained by constant adjustments of D; any deviation from strict equality would lead to one of the two previous results. It is also possible that there exist mutants having precisely the same growth characteristics as the parent variety (Novick & Szilard, 1950). Then $\lambda_m = \mu_m$, $L = K$, and coexistence is possible at any dilution rate; continued formation of mutants of this kind may lead to their displacing the natives entirely.

With this last possible exception, equations (5), (6*a*), (6*b*) are the only conditions which can lead to the successful growth of the foreign organisms, which then supersede the natives.

Suppose, by way of example, that the established organism in a culture has $\mu_m = 3$ hr.$^{-1}$ and that a mutant appears with $\lambda_m = 2{\cdot}5$ hr.$^{-1}$ and $L = \frac{3}{4}K$. Then

$$\frac{\lambda_m K - \mu_m L}{K - L} = 1.$$

If the culture is running with $D = 0{\cdot}5$ hr.$^{-1}$, by (6*a*) the mutant may establish itself. But if the dilution rate is $1{\cdot}5$ hr.$^{-1}$, it cannot. Yet either kind of organism could survive alone at either dilution rate. The results of Novick & Szilard (1950) indicate that such mutants, with $\lambda_m \leqslant \mu_m$ but $L < K$, do appear and can survive by reason of their faster growth at very low concentrations of limiting nutrilite.

The main restriction on the validity of the above reasoning is the assumption (iv) that the two kinds of organisms do not interact except in their competition for the limiting nutrilite; it is in this direction that the explanation of any *Gegenbeispiel* should first be sought. My colleague Mr R. Elsworth has experienced persistent contamination of *Bacterium cloacae* with *Pseudomonas pyocyanea* in continuous culture on a medium containing glucose, ammonia and sulphate as sole sources of carbon, nitrogen and sulphur. *P. pyocyanea* would scarcely grow alone on the medium. In this instance it is therefore likely that the products of metabolism (or lysis) of the native organisms encouraged the growth of the contaminants.

There is a simple general expression for the relation between x, y and t if (and only if) the two saturation constants are equal. We then have for all t

$$\frac{dx}{dt} = x\left(\mu_m \frac{s}{K+s} - D\right),$$

$$\frac{dy}{dt} = y\left(\lambda_m \frac{s}{K+s} - D\right),$$

whence, eliminating s,

$$\frac{d}{dt}(\mu_m \log y - \lambda_m \log x) = D(\lambda_m - \mu_m);$$

$$\left(\frac{y}{y_0}\right)^{\mu_m} \bigg/ \left(\frac{x}{x_0}\right)^{\lambda_m} = \exp\{(\lambda_m - \mu_m)\,Dt\}. \qquad (14)$$

That is, if the two saturation constants are equal, the successful variety is the one with the larger maximum growth rate. By comparing (4) and (5) it can also be seen that if the maximum growth rates are the same, the successful variety is the one with the smaller saturation constant, although in this case no such simple relation as (14) exists. Both of these results are true for any possible D.

An equation, equivalent to (14), for the competition between species has been given by Volterra (1931).

STATISTICS OF DISPLACEMENT

The preceding section overlooks the circumstance that bacteria are discrete particles, and that normally only very few foreign organisms will enter the culture at one time. The chance that a few organisms will be washed out of the vessel before they divide is not negligible, and so the conditions we have established are necessary but not sufficient to secure the success of the incomers.

In what follows I shall assume that only a single organism of a mutant or contaminant appears at first in the culture. There is no difficulty in extending the reasoning to multiplets.

I have shown elsewhere (Powell, 1956) that the essential condition for the steady growth of an organism in terms of its generation-time distribution is

$$\int_0^\infty e^{-D\tau} f_D(\tau)\, d\tau = \tfrac{1}{2},$$

where $f_D(\tau)$ is the frequency function of generation time (τ) under the conditions obtaining in the culture vessel; $f_D(\tau)$ will depend on $\tilde{s}$ and so on D. This equation implies that the probability that an organism chosen at random will terminate in the vessel is $\tfrac{1}{2}$; or, alternatively, that the expected number of the immediate progeny of an organism is 1; consequently, that the number of organisms will remain constant. Suppose now that a single mutant or contaminant appears, and that the generation-time distribution of its kind is $g_D(\tau)$. Write ϵ for the probability that an organism of the new variety will terminate in the vessel:

$$\int_0^\infty e^{-D\tau} g_D(\tau)\, d\tau = \epsilon. \tag{15}$$

Then we should expect the new variety to have a finite probability of multiplying successfully if $\epsilon > \tfrac{1}{2}$. If λ is the growth-rate constant of the new variety:

$$\lambda = \lambda_m \frac{\tilde{s}}{L+\tilde{s}},$$

we have

$$\int_0^\infty e^{-\lambda\tau} g_D(\tau)\, d\tau = \tfrac{1}{2},$$

and comparison with (14) shows that $\epsilon > \tfrac{1}{2}$ implies $D < \lambda$ and so is tantamount to the inequality (5). The calculation of the chances is complicated by the existence of correlations between the generation times of related organisms. By far the most important is the correlation between sisters (about $+0{\cdot}6$; Powell, 1955, 1956) and others are probably quite negligible in comparison. We need therefore to know the chance that one or both of a pair of sisters will terminate in the vessel. If $S_D(\tau_1, \tau_2)$ is the joint distribution of generation times for such pairs, the chance that both will terminate in the vessel is

$$\int_0^\infty \int_0^\infty e^{-D(\tau_1+\tau_2)} S_D(\tau_1+\tau_2)\, d\tau_1\, d\tau_2 = \eta^2, \text{ say.} \tag{16}$$

The chance that neither of a pair will terminate in the vessel is

$$\int_0^\infty \int_0^\infty (1-e^{-D\tau_1})\,(1-e^{-D\tau_2})\, S_D(\tau_1, \tau_2)\, d\tau_1\, d\tau_2 = 1 - 2\epsilon + \eta^2,$$

since $S_D(\tau_1, \tau_2)$ may be assumed symmetrical in its arguments, and

$$\int_0^\infty S_D(\tau_1, \tau)\, d\tau_1 = \int_0^\infty S_D(\tau, \tau_2)\, d\tau_2 = g_D(\tau).$$

And the chance that some one terminates in the vessel and the other is washed out is

$$1-(\eta^2+1-2\epsilon+\eta^2)=2(\epsilon-\eta^2).$$

Now let q_n be the chance that an organism of the new variety, if it does terminate in the vessel, has no progeny of the nth generation: $q_1=0$. This chance can be analysed into three components:

$$q_n=Aq_{n-1}^2+2Bq_{n-1}+C;$$

the first term is the chance that both daughter organisms terminate in the vessel but both fail to have progeny of the $(n-1)$th generation; the second that only one terminates in the vessel, and has no progeny of the $(n-1)$th generation; the third that neither terminates in the vessel. In our application, $A=\eta^2$, $B=\epsilon-\eta^2$, $C=1-2\epsilon+\eta^2$, and these are exclusive and exhaustive, so

$$q_n=\eta^2q_{n-1}^2+2(\epsilon-\eta^2)\,q_{n-1}+(1-2\epsilon+\eta^2). \tag{17}$$

We are interested in the probability that the foreign organism will still be represented in the vessel after many generation times; roughly, in $1-q_\infty$. It can be shown (see, for example, Feller, 1950) that the q in equations such as (17) have a limit, here given by the smaller root of

$$q_\infty=\eta^2q_\infty^2+2(\epsilon-\eta^2)\,q_\infty+(1-2\epsilon+\eta^2).$$

Hence $$q_\infty=1,$$

or $$q_\infty=(1-2\epsilon+\eta^2)/\eta^2.$$

If $\epsilon\leqslant\frac{1}{2}$, the second solution gives $q_\infty\geqslant1$ and only $q=1$ is admissible—the new variety is certain to fail. If $\epsilon>\frac{1}{2}$, the probability of success is given by the second solution, provided that the organism which first enters the vessel happens to terminate in it. Let us write ϵ_i for the chance that the foreign organism will terminate in the vessel. It may well happen that $\epsilon_i\neq\epsilon$, i.e. that the expectation for the first organism is not typical of its kind. For instance there may be a lag in the growth of an isolated contaminant or 'phenotypic delay' in a mutant (see, for example, Ryan & Wainwright, 1954). Then the probability that the foreign organism will have progeny after many generations is

$$\left.\begin{aligned} p_\infty&=\epsilon_i(1-q_\infty)=\epsilon_i\left(\frac{2\epsilon-1}{\eta^2}\right) && (\epsilon>\tfrac{1}{2}),\\ &=0 && (\epsilon\leqslant\tfrac{1}{2}).\end{aligned}\right\} \tag{18}$$

Of course, the growth of the new organism causes a change, very slight at first, in the cultural conditions and so in ϵ and η. But the number of native organisms is so large in practice that the new variety can multiply considerably without sensibly altering the conditions. By the time say 1000 organisms of the new variety are formed (if this ever occurs) the outcome is practically certain unless ϵ is only very little greater than $\frac{1}{2}$. For the probability of failure is then about q_∞^{500}; if ϵ is as small as 0·505, this is about 10^{-9}. But after this stage the foreign organisms can be treated as varying continuously in concentration, by the method of the previous section. Success or failure is determined before multiplication has proceeded far enough to disturb the original equilibrium; equation (18) shows that a new variety may fail to establish itself, no matter how well it is adapted to do so, and if ϵ is only a little greater than $\frac{1}{2}$, the chance of success is poor. Some calculations based on measured generation times (Powell, 1956) suggest that η will usually differ by no more than 2–3 % from ϵ.

Practical workers in this subject have commented on the infrequency of contamination; the above results accord with and explain this observation. But they also show that the fate of a mutant or contaminant is dependent on complex features of the growth process which often can only be known *post facto*; we cannot be sure that the mutability of a species is exhausted, nor legislate against unknown contaminants except by excluding them. It is, however, possible that effective precautions will be developed as experience increases; thus it may be possible to delay shut-down by an occasional temporary increase in dilution rate, or by injecting single doses of a bacteriostatic substance. As Maxon (1955) pointed out, a continuous culture on an industrial scale is economic only when it can be maintained for long periods.

The above reasoning relates to systems of continuous culture in which the dilution rate is fixed by the experimenter (Monod, 1950; Novick & Szilard, 1950). Another type of apparatus (e.g. Bryson & Szibalski, 1952) uses the optical density of the culture to control the dilution rate through a servo in such a way that the concentration of organisms remains constant. The chances for the first stages of growth of a foreign organism are the same in both systems. In the second, the subsequent behaviour depends on the yield constants and relative light-scattering power of the two varieties of organisms, and on the performance of the servo; moreover, D is a function of t during a period of changing population. The second system therefore requires a different form of treatment.

SYNOPSIS

Contaminant organisms or mutants which appear in a continuous culture can grow successfully only when their maximum growth rate and saturation constants stand in a certain relation to the constants of the native organisms and to the rate of flow through the culture vessel (equations (5), (6*a*), (6*b*)). If they do establish themselves, they displace the native organisms entirely. However, the foreign organisms or their progeny may happen to be washed out of the vessel, so that even when the necessary conditions are fulfilled, successful growth is still a matter of chance. The period of doubt is concentrated in the first few generations. The probability for success (equations (15), (16), (18)) depends on the distribution of generation times, and there are initial complications depending on the state of the foreign variety at the time of its appearance. If, under the conditions obtaining in the culture, the foreign variety has only a small advantage in growth rate over the natives, its chance of success is small.

Continued infection by contaminants or mutants of higher growth rate must lead to their ultimate success, but because the fate of any individual is uncertain they may not multiply appreciably until after a delay of indefinite length.

These results are true only under certain assumptions, of which the most restrictive is the postulate that there is no interaction between the two varieties of organisms other than their competition for the available nutrients.

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Two New Species of Yeasts from New Zealand

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SUMMARY: A new species of *Candida* was isolated from sphagnum litter overlying a peat bog. It was non-fermenting and assimilated glucose, sucrose, maltose, lactose, galactose and potassium nitrate. The proposed name is *Candida muscorum*. A new *Rhodotorula* species, which it is proposed to name *R. graminis*, was isolated from the leaves of pasture grasses. Cultures on solid media were reddish pink and fluid in consistency. Glucose, sucrose, galactose and potassium nitrate were assimilated.

In studying the characters of the two yeasts to be described the physiological properties were determined by the methods of Lodder & Kreger-van Rij (1952). Assimilation in their terminology, which is used, means utilization as sole carbon and energy source (for sugars) or as sole nitrogen source (for potassium nitrate).

A new yeast from moss overlying a peat bog

A large proportion of the yeasts isolated from the rotting layer of sphagnum overlying a peat bog at Paraparaumu in the Wellington district was found to be of a cream coloured, non fermenting species which produced pseudomycelium, but not ascospores, ballistospores or arthrospores. The isolates assimilated glucose, sucrose, maltose, lactose, galactose and potassium nitrate, and so did not correspond with any of the described species of *Candida* Berkhout. It is proposed to name this species *C. muscorum*.

Candida muscorum sp.nov. (Fig. 1)

Cellulae in culturis novis ovoidae (2·5–4) × (7–19) μ. Cultura nova in agaro Sabouraudi albida, valde fluida. Pseudomycelium interdum abundat. Fermentatio nulla. In medio minerali cum glucoso, saccharo, maltoso, lactoso (exigue), galactoso (exigue) crescit. Nitras kalicus assimilatur.

Cells from young cultures oval to long oval (2·5–4) × (7–19) μ. Growth on Sabouraud (glucose peptone) agar cream, fluid; mycelial tufts develop in the depths of aging cultures. Pseudomycelium may be weakly or strongly developed and is not characteristic in appearance. A capsule could not be demonstrated.

Fermentation: absent.

Sugar assimilation: Glucose + Lactose + (weak)
Sucrose + Galactose + (weak)
Maltose +

Assimilation of potassium nitrate: strongly positive.

A starch-like compound was not formed on the medium of Mager & Aschner (1947).

Further isolations of this species have been made from two forest soils from Chatham Island, 700 miles east of the South Island of New Zealand. The species dominated the yeast flora of both soils.

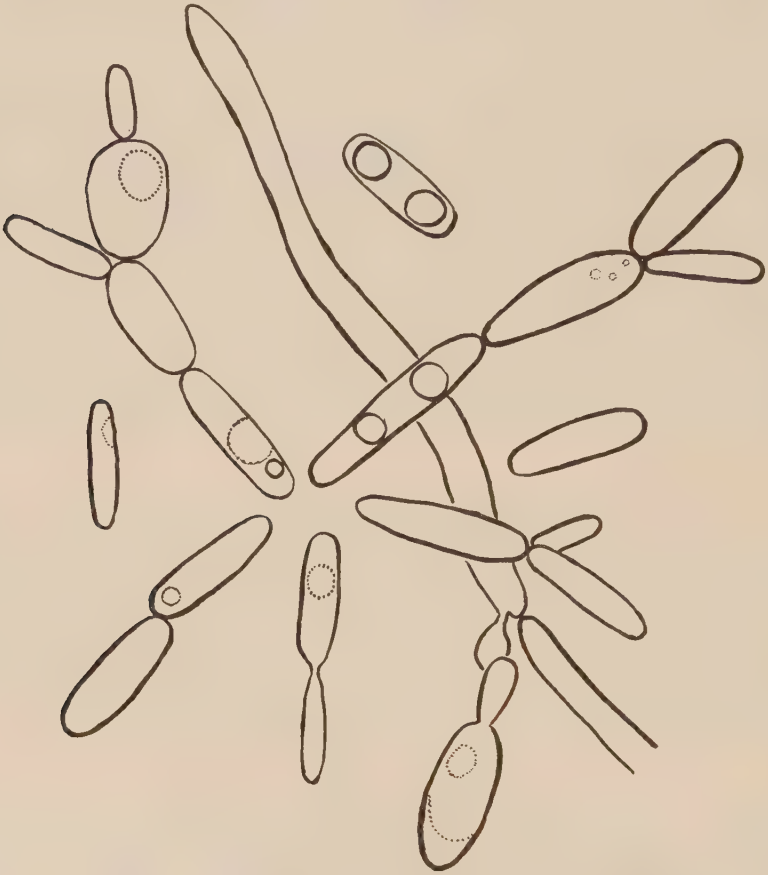

Fig. 1. Cells of *Candida muscorum* nov.sp. × 2,500.

A new yeast from pasture grasses

Isolations of yeasts from the leaf surfaces of pasture grasses (*Lolium perenne*, *Anthoxanthum odoratum* and *Agrostis tenuis*) collected in the North Island of New Zealand in summer were dominated by a new species of *Rhodotorula* Harrison which it is proposed to name *R. graminis*.

Rhodotorula graminis sp.nov. (Fig. 2)

Cellulae in culturis novis (4–5·5) × (4·5–7) μ. Cultura nova in agaro Sabouraudi subrubra, fluida. Pseudomycelium nullum. Fermentatio nulla. In medio minerali cum glucoso, saccharo, galactoso crescit. Nitras kalicus assimilatur.

Cells from young cultures oval to plump oval (4–5·5) × (4·5–7) μ. Intra- and extracellular fat drops are present, particularly in cultures on potato glucose agar. Growth on solid media pinkish red and fluid, running down the slope. There is little or no variation in the appearance of freshly isolated strains, although some, after long cultivation, may become mucoid rather than fluid.

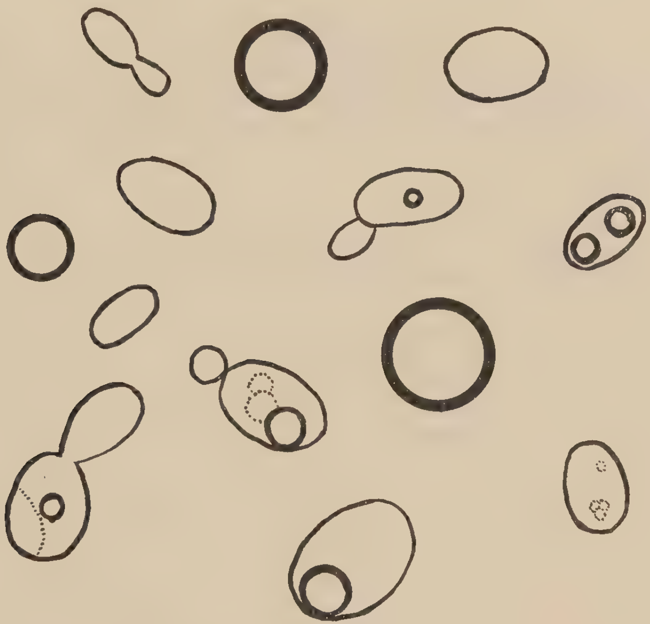

Fig. 2. *Rhodotorula graminis* nov. sp. Cells and extracellular fat drops; fat drops are heavily outlined circles. × 2,500

No pseudomycelium is formed.

Fermentation: absent.

Sugar assimilation: Glucose + Lactose –
Sucrose + Galactose +
Maltose –

Assimilation of potassium nitrate: strongly positive.

A starch-like compound was not formed on the medium of Mager & Aschner (1947).

Although the assimilation pattern of *Rhodotorula graminis* resembles that of *Sporobolomyces odorus* Derx there is no further resemblance between the species. Some strains of *S. odorus* were isolated from the grass samples upon which *Rhodotorula graminis* occurred in great numbers and these corresponded with the description given by Lodder & Kreger-van Rij (1952, p. 353). Cultures of *Sporobolomyces odorus* on solid media were cream to fawn and soft, not fluid, and at times powdery with ballistospores. No ballistospores or sterigmata could be demonstrated in any of the cultures of *Rhodotorula graminis*.

Cultures of the type strain of *Candida muscorum*, PS 2 from Paraparaumu, and of the type strain of *Rhodotorula graminis*, 2 K 53, have been deposited with the Yeast Division, Centraalbureau voor Schimmelcultures, Delft, Netherlands, and with the Brewing Industry Research Foundation, Nutfield, Surrey, England.

I am indebted to Dr N. J. W. Kreger-van Rij of the Yeast Division, Centraalbureau voor Schimmelcultures for confirmation of the validity of these species.

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Nutrition of Lactic Acid Bacteria Isolated from the Rumen

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SUMMARY: One hundred and seventeen strains of lactic acid bacteria from the rumen, representing four species of lactobacilli and four of streptococci, were examined for their requirements for vitamins. Of the lactobacilli only *Lactobacillus plantarum* (7 strains) was homogeneous in respect of its vitamin requirements. Within each of the other three species, strains differed in their requirements for individual vitamins, but on the whole the patterns of requirement were distinctive, and could be correlated with other specific characters. The vitamin requirements of three of the species (*L. casei*, *L. plantarum* and *L. acidophilus*) were broadly similar to those reported in a variety of non-rumen strains. Most of the rumen strains of *L. fermenti* required vitamin B_6 and riboflavin, in addition to the requirement for nicotinic acid, thiamine and pantothenic acid variously reported as typical of the species. *Streptococcus faecalis* and *S. liquefaciens* had distinctive patterns of vitamin requirements as did the unclassified group. The remaining species, *S. bovis*, had no requirement for exogenous vitamins under the anaerobic test conditions used. Under conditions of aerobic culture, however, strains of this species were found to require one or more vitamins for growth.

In a study of the types of bacteria normally present in large numbers (10^9–10^{11}/ml.) in the rumens of cows and calves, some 350 strains of streptococci and 300 of lactobacilli were isolated; about a quarter of these were taken as representative, and identified (Perry, Wilson, Newland & Briggs, 1955; Perry & Briggs, 1957). The present study was carried out to determine the patterns of vitamin requirements in these rumen organisms and to establish to what extent these patterns are characteristic of the species as differentiated on the basis of bacteriological tests. A longer-term objective is to study symbiotic interrelationships between the more important rumen species, and the nutritional factors which may determine the equilibrium between the assorted populations present.

The nutrition of the lactic acid bacteria has been widely studied, and the vitamin requirements of representative strains have been worked out in detail (cf. Snell, 1948; Rogosa, Wiseman, Mitchell, Disraely & Beaman, 1953). So far, however, very few strains from the rumen have been included in these studies. Jensen, Smith, Edmondson & Merilan (1956) identified eight species of lactobacilli present in the bovine rumen at concentrations of 10^6 or 10^7/ml. They concluded, on the basis of classical bacteriological tests, that none was indigenous to the rumen, and implied that their requirements for vitamins and amino acids did not require investigation, being already 'well known'. The implication is perhaps somewhat misleading, giving an impression that characteristic nutritional requirements can be precisely stated for each of the species. Against this, Russell, Bhandari & Walker (1954), from a study of the vitamin requirements of lactic acid bacteria associated with brewery products, concluded that the vitamin requirements of different strains of any one species varied widely, though strains isolated from the same source tended to have the same requirements. In comparing the conclusions of different workers about the nutritional requirements of the lactobacilli one must bear in mind that these requirements can depend rather critically upon the conditions of test; they may be non-specific and sometimes more apparent than real (cf. Snell, 1948). For example, the absence of a requirement for vitamin B_{12} in all 19 strains of *Lactobacillus acidophilus* examined in the present study contrasts with the finding by Rogosa *et al.* (1953) that a requirement for this vitamin was an important characteristic of the species, and may perhaps be related to the more strictly anaerobic conditions under which our tests have been done. There is, however, a broad area of agreement as to the requirements of certain species for particular vitamins, and it would seem that, despite all the factors making for variability, these nutritional requirements, under strictly defined conditions, are remarkably stable, and might well prove of value as an aid to classification.

The nutrition of several species of the genus *Streptococcus* has been studied in some detail, notably by Niven and his co-workers. Several strains of *S. faecalis*, *S. liquefaciens*, *S. durans* and *S. zymogenes*, all members of Lancefield's serological group D, were found (Niven & Sherman, 1944) to have broadly similar vitamin requirements: all required biotin, nicotinic acid, pantothenic acid and vitamin B_6, and all except two required riboflavin. This pattern of vitamin requirement has been confirmed for *S. faecalis* by Nevin (1954). Niven & Sherman (1944) concluded that, in their growth-factor requirements, the enterococci form a homogeneous group, but more recently they have recognized differences in the nutritional requirements of these species (Lake, Deibel & Niven, 1957; and see below). Although belonging to the same serological group D, *S. bovis* is apparently unique in the simplicity of its vitamin requirements (Niven, Washburn & White, 1948). Most strains were reported to have an absolute requirement for biotin only, although thiamine, nicotinic acid and pantothenic acid were stimulatory and proved essential on prolonged serial subcultivation.

METHODS

The media and techniques used for the isolation and purification of the cultures have been described elsewhere (Perry *et al.* 1955; Perry & Briggs, 1957). Most of the organisms were isolated from the rumen contents of cows or calves, though a few were obtained from sheep (K. D. Perry, unpublished). Seventy-six strains of lactobacilli, representing four species, and 37 strains of streptococci representing three species and one unclassified group, were grouped according to their patterns of vitamin requirements.

Cultural characteristics

Lactobacilli. The results of the cultural tests used to differentiate these organisms into the four species are set out in Table 2. Serological tests confirmed this classification in representative strains of three of the species. This was not possible with *Lactobacillus acidophilus*, for which no antiserum was available.

Streptococci. The characteristics of the streptococci are shown in Table 3. All the isolates belonged to Lancefield's serological group D, within which they were divided according to their different cultural reactions into three distinct species and one uniform unclassified group.

Vitamin requirements

For the determination of vitamin requirements the following procedure was adopted. Freeze-dried cultures were seeded into a peptone + yeast extract + meat extract + sucrose + salt medium (Hirsch & Grinsted, 1954), from which inoculations were made to tubes containing 10 ml. of 'complete' medium, of the composition shown in Table 1.

Table 1. *Composition of the 'complete' medium*

Solution of vitamin-free acid-hydrolysed casein (see text)	100 ml.	Xanthine	5 mg.
		Solution of mineral salts (see text)	10 ml.
Glucose	10 g.	Tween 80	1 ml.
Sodium-β-glycerophosphate	5 g.	Pyridoxal hydrochloride	2 mg.
Sodium acetate (hydrated)	4 g.	Nicotinic acid	1 mg.
K_2HPO_4	2 g.	Calcium pantothenate	1 mg.
Potassium citrate (hydrated)	0·6 g.	Thiamine hydrochloride	1 mg.
Ascorbic acid	0·5 g.	*p*-Aminobenzoic acid	0·5 mg.
Cysteine hydrochloride	50 mg.	Riboflavin	0·5 mg.
Adenine	5 mg.	Folic acid	0·25 mg.
Guanine hydrochloride	5 mg.	Biotin	$10\,\mu$g.
Uracil	5 mg.	Vitamin B_{12}	$1\,\mu$g.

This mixture was made up in boiled distilled water to 1000 ml., the pH value having been adjusted to 6·5–6·7.

The solution of vitamin-free acid-hydrolysed casein used in the 'complete' medium (Table 1) was made up as follows: 50 g. 'vitamin-free' hydrolysed casein (Allen and Hanbury Ltd.) were dissolved in 700 ml. water. The pH value was adjusted to 3·8 with acetic acid, 10 g. activated charcoal (Sutcliffe and Speakman Ltd., Grade 5) added, the mixture stirred for 1 hr. and filtered. The filtrate was adjusted to pH 6·5 with 10 N-KOH, treated with a further 5 g. charcoal, and filtered again. Finally, 1 g. DL-tryptophan, 200 mg. each of L-tyrosine and DL-β-phenylalanine and 100 mg. each of L-proline and L-histidine were dissolved into the filtrate, and the solution diluted to 1 l. with water.

The solution of mineral salts used in the 'complete' medium (Table 1) contained (g.): 20, $MgCl_2.6H_2O$; 5, $CaCl_2$ (anhyd.); 0·5, $FeCl_3.6H_2O$; 0·5, $ZnSO_4.7H_2O$; 0·5, $MnSO_4.4H_2O$; 0·25, $CoCl_2.6H_2O$; 0·25, $CuSO_4.5H_2O$; 0·25, VSO_4; 0·25, Na_2MoO_4 dissolved in 1 l. distilled water with addition of N-H_2SO_4 to clear.

Table 2. *Cultural characteristics of the lactobacilli tested**

	L. fermenti	*L. acidophilus*	*L. casei*	*L. plantarum*
No. of strains tested	33	19	17	7
Production of gas from glucose	+	–	–	–
Production of NH_3 from arginine	+	–	–	–
Growth at 15°	+	–	+	+
45°	+	+	+	+
48°	– (20)	–	– (12)	–
Growth with NaCl 4 %	+ (22)	+	+	+
6 %	–	–	+	+ (4)
Growth with Teepol 0·05 %	NT	+	NT	NT
0·1 %	–	NT	–	+
Growth with $KTeO_3$ 0·01 %	+	NT	NT	NT
Action in yeast extract glucose litmus milk	sl. A	AC (3 days)	AC (2 days)	AC (5 days)
Fermentation of:				
Amygdalin	–	+ (15)	+	+
Arabinose	+	–	– (11)	+
Cellobiose	–	+	+	+
Dextrin	NT	+	NT	NT
Glycerol	–	–	–	+ (4)
Inositol	–	– (13)	+ (10)	–
Lactose	+	+	+	+
Maltose	NT	+	NT	NT
Mannitol	–	–	+	+
Melezitose	–	–	+	+
Melibiose	+ (18)	+	–	+
Raffinose	+	+	–	+
Rhamnose	–	–	– (16)	–
Salicin	– (27)	+	+	+
Sorbose	–	–	+	+
Sucrose	+	+	+	+
Trehalose	–	+ (12)	+	+
Hydrolysis of aesculin	NT	+	NT	NT

* Partly from Perry & Briggs (1957).
Figures in parentheses represent the number of strains giving the reaction indicated.
sl. A: slight acidity; AC: acidity and clotting; NT: not tested.

The complete medium (Table 1) allowed abundant growth of all the isolates. The method of testing for vitamin requirements was to make, in parallel, three serial subcultures in: (*a*) complete medium; (*b*) complete medium modified by the omission of one of the eight vitamins—riboflavin, calcium pantothenate, nicotinic acid, pyridoxal hydrochloride, folic acid, biotin, thiamine hydrochloride or vitamin B_{12}; (*c*) complete medium from which all eight vitamins were omitted simultaneously. Requirements for *p*-aminobenzoic acid were not investigated, and it was included in all the test media. The cultures were incubated at 39° for 20 hr. in an atmosphere of H_2 90 % (v/v) + CO_2 10 % (v/v); a one-drop (*c*. 0·5 %) inoculum was transferred at each subculturing. Growth was assessed by visual observation of the tubes at 20 hr., and was scored as: – (no growth), ± (slight growth), +, ++ or +++.

Table 3. *Cultural characteristics of the streptococci tested.*

	S. bovis	*S. liquefaciens*	*S. faecalis*	Unclassified
Number of strains tested	26	5	2	8
Serological (precipitin) reaction with antisera of:				
Groups A–C, E–N	–	–	–	–
Group D	+	+	+	+
Growth at 45°	+	+	+	+
with NaCl, 6·5 %	–	+	+	+
at pH 9·6	–	+	+	+
Survival at 60° for 30 min.	–	+	+	+
Liquefaction of gelatin	–	+	–	–
Hydrolysis of arginine	–	+	+	+
Hydrolysis of starch	+	–	–	–
Haemolysis in horse blood agar	α	sl. α	sl. α	α
Action in litmus milk	sl. A	RC, pept.	ARC	ARC
Growth with 0·04 % (w/v) $KTeO_3$:	NT	NT	+	–
Fermentation of:				
Arabinose	–	–	–	–
Glycerol	–	+	+	+
Inulin	+	–	–	–
Mannitol	–	+	+	–
Melezitose	NT	NT	+	–
Melibiose	NT	NT	–	+
Raffinose	+	–	+	+
Sorbitol	–	+	+	–
Sucrose	+	+	+	+
Trehalose	–	+	+	+

sl. A: slight acidity; ARC: acidity, reduction and clotting; RC, pept.: reduction and clotting, followed by peptonization; NT: not tested.

We found that with most organisms the vitamin requirements were sharply defined and unequivocal. In the absence of a required vitamin there was no visible growth in the second or third subculture. An exception was the finding that growth of a few strains of *Lactobacillus plantarum* in the absence of vitamin B_6 was severely limited, but continued at ± through several transfers. Many of the tests were repeated, including all of those indicating the less common patterns of vitamin requirements. For example, the absence of a requirement for riboflavin in two strains of *L. fermenti* was confirmed in parallel tests with strains which required riboflavin and in freshly prepared test medium.

Three of the cultures of lactobacilli appeared to have no requirement for vitamins, and were found on further investigation to be contaminated with *Streptococcos bovis.*

RESULTS AND DISCUSSION

Lactobacilli

The vitamin requirements found in the four species are shown in Table 4.

Lactobacillus casei. Several reports on the vitamin requirements of *L. casei* differ only in detail. Rogosa *et al.* (1953) found that a requirement for riboflavin, pantothenic acid, nicotinic acid and folic acid, and poor growth without vitamin B_6, was characteristic of the species as represented by some hundreds of oral strains. This pattern is similar to that of most of our rumen strains, except that in all of these the need for vitamin B_6 was absolute, and that for some of them folic acid was not required. Snell (1945) and Shankman *et al.* (1947) reported an additional requirement for biotin. The absence of this requirement in all the strains tested by Rogosa *et al.* (1953) and by ourselves is no doubt explained by the presence of oleate (as Tween 80) in our culture media, since it is known that for many lactic acid bacteria, oleate and biotin are physiologically equivalent, although for others (e.g. *L. acidophilus*) both substances are required (Kitay & Snell, 1950). Our rumen isolates were broadly similar in their cultural reactions, but Rogosa *et al.* (1953), in a more searching comparison of the cultural reactions of their oral strains, recognized three varieties: *L. casei* var. *casei* var.nov., which does not ferment rhamnose but ferments lactose; *L. casei* var. *alactosus* var.nov., which ferments neither rhamnose nor lactose; *L. casei* var. *rhamnosus* var.nov. which ferments both rhamnose and lactose. Within these three varieties there was considerable variation in fermentation reactions, though mostly in respect of substances which were fermented either weakly or not at all. It would seem then that these oral strains of *L. casei* were heterogeneous on the basis of their cultural reactions, albeit identical in their vitamin requirements. Conversely, in our rumen strains we have recognized two distinct patterns of vitamin requirement but no determining differences in cultural reactions, except possibly in the fermentation of rhamnose by one strain. Such differences as were evident—(growth at 48°, fermentation of arabinose and inositol) could not be correlated with the different patterns of vitamin requirement. For comparison, we examined four stock strains of *L. casei*, originally isolated from milk or cheese. Two of these strains belonged to the serological group B of Sharpe & Wheater (1957) and the other two strains to the same authors' serological group C. Three of the four strains had the same vitamin requirements as the rumen group G (Table 4), and one the same as group H. A point of interest here is the overlapping of the subdivisions according to serological and nutritional features.

Lactobacillus fermenti. Of 148 oral strains of *L. fermenti* studied by Rogosa *et al.* (1953), 95 % or more required thiamine, nicotinic acid and pantothenic acid. Shankman *et al.* (1947) reported that *L. fermenti* 36 needed only nicotinic acid and pantothenic acid, although Snell (1945) had found additional requirements for thiamine and biotin. All our rumen strains required nicotinic acid, pantothenic acid and thiamine; none required biotin, but 31 of the 33 required riboflavin and 26 required vitamin B_6. Our isolates were alike in most of their cultural characteristics, and we were content to assign them all to one group, but if greater significance were attached to small differences in fermentative abilities, further subdivision to 'varieties' would have been possible. From our experience we have preferred the more conservative approach, whereas Rogosa *et al.* (1953) were prepared to subdivide their strains into 12 groups on the basis of what we would have considered trivial differences in fermentation patterns. For comparison with our rumen strains we determined the vitamin requirements of four stock strains of *L. fermenti* originally isolated from cheese. Two of these strains, like the oral strains of Rogosa *et al.* (1953) required

Table 4. *Vitamin requirements of lactobacilli*

Species	Requirement for								No. of strains	Nutritional pattern
	Riboflavin	Nicotinic acid	Pantothenic acid	Thiamine	Vitamin B_6	Vitamin B_{12}	Biotin	Folic acid		
Lactobacillus fermenti (33 strains)	+	+	+	+	+	–	–	–	24	A
	+	+	+	+	–	–	–	–	7	B
	–	+	+	+	+	–	–	–	2	C
L. acidophilus (19 strains)	+	+	+	+	–	–	+	+	10	D
	+	+	+	–	–	–	+	+	2	E
	+	+	+	–	–	–	–	+	7	F
L. casei (17 strains)	+	+	+	–	+	–	–	+	12	G
	+	+	+	–	+	–	–	–	5	H
L. plantarum (7 strains)	–	+	+	–	+*	–	–	–	7	I

+ : signifies absolute requirement.
* But see text, p. 280.

Table 5. *Vitamin requirements of streptococci*

Species	Requirement for								No. of strains
	Riboflavin	Nicotinic acid	Pantothenic acid	Thiamine	Vitamin B_6	Vitamin B_{12}	Biotin	Folic acid	
Streptococcus bovis	–	–	–	–	–	–	–	–	26
S. liquefaciens	+	+	+	–	+	–	–	–	5
S. faecalis	+	+	–	–	+	–	–	–	2
Unclassified	–	+	+	–	+	–	–	+	8

+ : signifies absolute requirement.

thiamine, nicotinic acid and pantothenic acid. One had a further requirement for riboflavin, as did the rumen group B (see Table 4), whereas the fourth had an extra requirement for vitamin B_6, like the rumen group C. As with *L. casei*, we were unable to correlate the different nutritional patterns within *L. fermenti* with any differences in cultural characteristics. A similar absence of detailed correlation between nutritional requirements and fermentation reactions is apparent in the findings of Rogosa *et al.* (1953).

Lactobacillus acidophilus. All of our strains of *L. acidophilus* required riboflavin, nicotinic acid, pantothenic acid and folic acid. In this they resemble the oral strains examined by Rogosa *et al.* (1953), which however also required vitamin B_{12}, vitamin B_6 being stimulatory. No need for these latter two vitamins was evident in our rumen isolates, two of which, however, had an additional requirement for biotin, and ten for biotin + thiamine. Kitay & Snell (1950) found, in agreement with Rogosa *et al.* (1953), that vitamin B_{12} or deoxyribosides were essential for growth in 9 of 10 strains of *L. acidophilus*. It may well be that under different conditions of test a requirement for this vitamin would be manifest in our rumen strains. Our test medium was in fact virtually free from vitamin B_{12}; its composition was essentially the same as that of the medium we use routinely in the assay of vitamin B_{12} with *L. leichmannii* ATCC 4797, which contains less than 1 $\mu\mu$g. vitamin B_{12}/ml. Kitay & Snell (1950) also reported a requirement for pantetheine ('*Lactobacillus bulgaricus* factor') in 6 of 9 strains of *L. acidophilus*. None of our rumen isolates, nor any of the oral lactobacilli studied by Rogosa *et al.* (1953), showed a specific requirement for *L. bulgaricus* factor. The oral strains examined by Rogosa *et al.* had uniform requirements for vitamins, and were similar in their cultural reactions. Our rumen strains, on the other hand, were less consistent in their fermentation reactions, and fell into three distinct subgroups according to their different patterns of vitamin requirements.

Lactobacillus plantarum. For our rumen strains nicotinic acid and pantothenic acid were required, and without added vitamin B_6 growth was severely limited. Rogosa *et al.* (1953) recognized two distinct nutritional types in their oral isolates. One required nicotinic acid and pantothenic acid; the other required nicotinic acid and riboflavin, and grew very poorly in the absence of pantothenic acid. The strains which required riboflavin were further subdivided into two groups on the basis of marked differences in their fermentation reactions. Those which required nicotinic acid and pantothenic acid, however, had uniform and distinctive fermentation reactions, and Rogosa *et al.* (1953) gave important reasons for concluding that these latter strains are identical with the *L. arabinosus* of Fred, Peterson & Anderson (1921). This they claimed to be a species quite distinct from *L. plantarum*. Whether or not this is so, the isolates resemble our rumen strains very closely, both in cultural characteristics and vitamin requirements. On the question of the status of *L. arabinosus* as a distinct species we prefer to reserve our opinion as all our rumen strains give a positive reaction with the group D (*L. plantarum*) antiserum of Sharpe & Wheater (1957).

Streptococci

The vitamin requirements found for the streptococci are shown in Table 5.

Streptococcus bovis. None of the 26 strains tested required exogenous vitamins, though 5 grew poorly in the absence of thiamine. These findings contrast with those of Niven *et al.* (1948), most of whose strains required only biotin, but were said (somewhat paradoxically) to require also thiamine, pantothenic acid and nicotinic acid on prolonged subcultivation. To allow a closer comparison of our findings with those of Niven *et al.* (1948), we repeated several of the tests, incubating the cultures aerobically. Growth under these conditions was less vigorous, and several patterns of vitamin requirement became apparent. Thus, nine of our strains failed to grow without nicotinic acid, and three without nicotinic acid and biotin. One required nicotinic acid, biotin and thiamine; four required biotin and thiamine and one required biotin. These aerobic requirements were not entirely constant and are presumably not absolute: they are evidently imposed more by the conditions of culture than by an inherent inability to synthesise these essential metabolites. Our rumen strains of *S. bovis* were uniform in their cultural reactions, except that two strains fermented trehalose, one arabinose, and one strain fermented both sugars. Their cultural characteristics are very similar to those of the strains described by Niven *et al.* (1948), and the organisms may well be identical, despite the inconclusive evidence of the nutritional tests.

Streptococcus liquefaciens. The five strains of *S. liquefaciens* required riboflavin, nicotinic acid, pantothenic acid and vitamin B_6, whether they were grown aerobically or anaerobically; they were identical also in their cultural reactions. Niven & Sherman (1944), who used a medium free from oleate, found that biotin also was required. These vitamin requirements recall those of *L. casei*, and are indeed the same as those of our *L. casei* subgroup H (Table 4).

Streptococcus faecalis. Two of the streptococci were identified as *S. faecalis* and required vitamin B_6, nicotinic acid and riboflavin. The absence of a requirement for folic acid accords with the findings of Lake *et al.* (1957); these workers used this criterion to differentiate *S. faecalis* and its varieties from *S. faecium* and *S. durans*. By implication, strains of *S. faecalis* reported as requiring folic acid (Niven & Sherman, 1944) should be re-assigned to *S. faecium*; but so much of the classical work on folic acid has been carried out with deficient strains of '*S. faecalis*' that any such belated revision of the nomenclature would seem likely to create misunderstanding.

Unclassified strains. The eight strains examined required nicotinic acid, pantothenic acid, vitamin B_6 and folic acid, and were identical in their cultural reactions. Absence of growth in presence of 0·04 % (w/v) $KTeO_3$ and requirement for folic acid suggested that they did not belong to the *Streptococcus faecalis* group; the remaining group D species, *S. bovis*, *S. durans* and *S. faecium* were excluded respectively, on the results of tests for heat survival and growth at pH 9·6 or in 6·5 % NaCl; haemolysis in horse blood agar and fermentation of sucrose and raffinose; fermentation of arabinose and raffinose. These eight strains resemble certain unclassified isolates from the gut of the pig (Fewins, Newland & Briggs, 1957).

CONCLUSIONS

Of the four species of rumen lactobacilli examined only *Lactobacillus plantarum* (represented by 7 strains) was homogeneous in respect of its vitamin requirements. Within each of the other three species, strains differed in respect of their requirements for individual vitamins, but on the whole the patterns of requirement appeared to be characteristic, in that they were correlated with other specific characters. On the wider question of the extent to which these nutritional patterns can be held to differentiate the species and subspecies it will clearly be necessary to investigate a fuller range of representative strains.

The vitamin requirements of three of the species of rumen lactobacilli, namely *Lactobacillus casei*, *L. plantarum* and *L. acidophilus*, were broadly similar to those reported for a number of non-rumen strains, as also were most of the cultural characteristics, and there seems little reason to suppose that the rumen strains are in any way different from strains previously isolated from other sources. Rogosa *et al.* (1953) came to the same conclusion with regard to their oral strains. There is in the findings of Russell *et al.* (1954) the suggestion that lactobacilli isolated from sour beers and brewers' yeasts are different from their counterparts in milk in that none of 32 isolates, representing 8 species, required folic acid or biotin. This may be so but it seems to us possible that the method of analysis adopted by Russell *et al.* would not have brought to light any requirements for these more 'potent' vitamins. We found in general that requirements for thiamine, riboflavin, nicotinic acid and pantothenic acid were manifest at the first transfer into deficient media, whereas requirements for vitamin B_6, folic acid and biotin were not apparent until the second or even the third transfer. To assess the vitamins as 'essential', 'stimulatory' or 'non-essential' on the evidence of a single transfer into the deficient media, as was done by Russell *et al.* (1954) and by several other workers (e.g. Anderson & Elliker, 1953) seems to us therefore to be less than adequate, and perhaps misleading. One suspects that vitamins in the 'stimulatory' category would be judged 'essential' on further investigation, and that certain 'non-essential' vitamins might in fact be required.

Most of our rumen strains of *Lactobacillus fermenti* required riboflavin and vitamin B_6 in addition to the requirement for nicotinic acid, thiamine and pantothenic acid reported as typical of the species by Rogosa *et al.* (1953). Under our conditions of test requirements for riboflavin or vitamin B_6 were also found in strains of *L. fermenti* from cheese. Thus there seems no reason to suppose that the rumen strains are unique in their vitamin requirements.

In general, our assessment of the vitamin requirements of *Streptococcus faecalis* and *S. liquefaciens* confirms the findings of Niven and his co-workers. In *S. bovis*, however, we found no absolute requirement for vitamins under our conditions of test. The unclassified strains were homogeneous in respect of their vitamin requirements and cultural characters, which together differentiated them from named streptococcal species. From a comparison of cultural characteristics, it seems likely that these strains may be the same as the unclassified isolates of Fewins *et al.* (1957) from the gut of the pig.

Our experience in carrying out this limited survey of the rumen lactic acid bacteria has impressed us with the potential value of information on comparative nutrition in studies of bacterial taxonomy. We have at this Institute a collection of fully classified cultures, representative of all the accepted species in the genus *Lactobacillus*, and we plan to extend the investigation reported in this paper to include several strains of each of these species.

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The Precipitating Antigen of *Streptococcus pyogenes* Type 4

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SUMMARY: The antigen found in acid extracts of some type-4 Group-A streptococci which is responsible for precipitation with specific antisera is resistant to digestion with trypsin and pepsin when in the cell. It becomes sensitive to these enzymes after extraction with boiling HCl. It is not an M antigen but resembles a T antigen although it is more heat-stable at low pH values than the T antigen of most other types.

Another precipitating antigen, common in freshly isolated type-4 strains but not restricted to them, is described and referred to as 'B'. Its role as a blocking agent in inhibiting the formation of M antibody is discussed.

The M antigen is now accepted as the basis for differentiating the serotypes of *Streptococcus pyogenes* (Lancefield's Group A). This antigen, which is a protein sensitive to digestion by trypsin and pepsin, has two important characters: (1) when extracted with hot acid by the Lancefield method (Swift, Wilson & Lancefield, 1943) it precipitates specifically with absorbed antisera prepared against strains of the same M type; (2) antibodies to M antigen will specifically protect mice against infection with streptococci of the same type (Lancefield & Perlmann, 1952*a*). The addition to fresh human blood of rabbit serum containing an M antibody also enhances the bactericidal property of the blood for strains possessing that particular M antigen (Maxted, 1956).

It is difficult to prepare satisfactory anti-M serum for streptococci of type 4; the precipitate obtained even with extracts of the vaccine strain is usually weak as compared with the homologous precipitate of other types. Moreover, Maxted (1956) found that a type-4 antiserum which gave this weak precipitation did not enhance the bactericidal property of human blood against type-4 organisms. There is no evidence about mouse protection, since none of the strains so far tested have been sufficiently virulent to be tested in mice.

In certain batches of anti-M sera cross-reactions have been noted in precipitin tests between types 4, 26, 29 and 46. The reaction was of the strength of a usual type-4 precipitation and did not approach that given by normal type-26, 29 or 46 M-antigen extracts with their homologous antisera. Since types 4, 26, 29 and 46, together with type 24, possess a common T antigen (Stewart, Lancefield, Wilson & Swift, 1944) it seemed possible that the cross-reaction might be due to a T antigen which survived extraction by hot acid, even though the T antigen of most types is destroyed by this treatment. If this were so, the rather poor precipitin reaction obtained between type-4 Lancefield extracts and type 4 antiserum might be due, not to an M antigen, but to a T antigen. This appears to be the case.

METHODS

Organisms used. The following strains of *Streptococcus pyogenes* were used:

Type	Strains	Source of strain
4	T4/95/Rb5/7 (also known as 4990)	Original Griffith Type-4 strain passed through 95 mice and 5 rabbits by Lancefield
4	R54.2692 R54.2707 R54.2604 R55.17	Strains freshly isolated from throats of scarlet fever patients
1	SF130/13	Original Griffith Type 1 passed through 13 mice by Lancefield
6	S43 glossy	Isolated by Dochez, Avery & Lancefield
6	S43/78	Isolated by Dochez, Avery & Lancefield, passed through 75 mice by Lancefield; through 3 mice at Colindale
11	T11/54/4	NE73T passed through 58 mice by Lancefield
Provisional type B3264	C632	Lancefield (originally from Griffith)

Cultivation of type-4 strains. Type-4 strains were grown in a modified Todd–Hewitt broth for 18 hr. at 37°. This temperature was found to favour production of the antigen responsible for both agglutination and precipitation.

Recognition of antigens

Slide agglutination. Overnight cultures in 5 ml. broth were centrifuged and all but 0·5 ml. of the supernatant fluid removed. Pancreatic extract (Cole & Onslow) was added, the pH value adjusted to 8, and the suspensions of organisms then incubated at 37° for 2 hr. This treatment renders the suspensions smooth and usable for agglutination and also destroys the M antigen when present.

Lancefield extracts for M antigen. The cocci from 50 ml. of overnight broth cultures were suspended in 0·4 ml. of 0·2N-HCl and placed in a boiling water-bath for 10 min. After cooling the mixture was neutralized and the supernatant fluid used as the extract. Precipitin tests were carried out in capillary tubes (Swift *et al.* 1943).

Pakula extracts for T antigen (Pakula, 1951). Cocci from 100 ml. of an overnight broth culture were washed in saline, suspended in 2·7 ml. of buffer, pH 7·8, and heated at 80° for 10 min. The suspension was cooled and 0·3 ml. of pancreatic extract was added, the pH value adjusted to 8 and the mixture held at 50° in a water-bath for 2 hr. The suspension was then spun, the supernatant removed and adjusted to pH 2 with N-HCl. The T antigen precipitated at this pH value when left overnight in the cold. It could then be removed from the centrifuged supernatant fluid and redissolved in 0·1 ml. buffer at pH 7·8. The extracts were tested in capillary tubes in the same way as Lancefield extracts.

Treatment of antigens with trypsin and pepsin. Pancreatic extract was used as a source of trypsin, but all the more important results were confirmed by using a solution of crystalline trypsin (Armour and Co., Ltd.); a 0·0007 % (w/v) solution of crystalline trypsin was found to have an effect equivalent to the solution of pancreatic extract used. Crystalline pepsin (Armour and Co., Ltd.) was used in 10 % (w/v) solution.

When Lancefield extracts were treated with trypsin or pepsin, 0·04 ml. of the enzyme solution was added to 0·2 ml. of the extract; the pH value was adjusted to 8 for trypsin and 2·5 for pepsin. The extracts were held at 37° for 2 hr., the pH value re-adjusted to neutral, and the extract tested for precipitation with appropriate antisera.

When enzyme treatment was carried out on the streptococcal cells, cocci from an overnight culture in 50 ml. broth were suspended in 0·7 ml. of appropriate buffer and 0·3 ml. of enzyme solution added. The suspensions were held at 37° for 2 hr. The cocci were then separated from the buffer by centrifugation, extracted by the Lancefield method and tested in the usual way. In all experiments controls were included, using buffer solution without the addition of enzyme.

Preparation of antisera. Antisera were prepared by the inoculation of rabbits with whole cocci killed by heat at 56° for anti-M or with cocci digested with trypsin, washed and killed with formalin for anti-T sera. In either case growth from an overnight broth culture was concentrated 10 times for use as vaccine. After a sensitizing dose of 0·5 ml. the rabbits were given 1 ml. doses on 3 consecutive days followed by 4 days rest. This dosage was repeated weekly, usually for 4–5 weeks.

The type-4 antiserum used in most of this work was prepared against strain 4990.

Absorption of antisera. For agglutination and precipitin tests the antisera were absorbed with cocci of type 6, strain S 43 glossy, to remove group and non-specific antibodies. Antisera made from freshly isolated type-4 strains had also to be absorbed with a type-11 strain, T 11/54, to remove the B antibody, which is described more fully in the text. B antibody was not present in sera prepared against strain 4990.

Preparation of fractions of type-4 streptococci for absorption of sera

T fraction. The absorbing fraction was the precipitate brought down at pH 2 in a Pakula extract. The cocci from 500 ml. broth incubated for 48 hr. yielded sufficient crude T antigen to absorb all the T antibody from 0·3 ml. of type-4 antiserum.

'M' fraction. Cocci were extracted by the Lancefield method; 2·5 vol. of absolute ethanol were then added to the extract. This precipitated a fraction which on redissolving gave a precipitation reaction with type-4 antiserum but contained no group polysaccharide. The extract was reprecipitated three times with ethanol and the final precipitate was used for absorption of serum. Cocci grown in 500 ml. broth for 48 hr. yielded an absorbing dose for 0·3 ml. of serum.

Strain 4990 was used in the preparation of both fractions. Absorptions were carried out by mixing the antiserum with the antigen fraction, incubating for 2 hr. at 37° and then placing the mixture in the cold overnight before testing. In all cases controls were included.

RESULTS

The effect of trypsin and pepsin on the type-4 precipitating antigen

The effect of trypsin and pepsin was tested on Lancefield extracts and live streptococci of types 4 and 1. Whereas trypsin digestion destroyed the serological activity of the type-4 antigen extracted by hot hydrochloric acid, it failed to destroy that of the antigen extracted at 50° or that from whole cocci treated with trypsin before extraction (Table 1). The type-1 antigen was destroyed by trypsin both in the extract and the cocci.

Table 1. *The effect of trypsin and pepsin on the type-4 precipitating antigen*

	Treatment of cocci or extracts				
	None	Trypsin digestion at pH 7·8	Buffer at pH 7·8	Pepsin digestion at pH 2·5	Buffer at pH 2·5
Acid extracts made from untreated cocci					
Type 4 extracted at 100°	++/++	−/−	+/+	−/−	+/+
Type 4 extracted at 50°	+/+	tr/+	+/+	N.D.	N.D.
Type 1 extracted at 100°	++/++	−/−	+/+	−/−	++/++
Cocci treated before acid extraction					
Type 4	++/++	+/++	++/++	−/−	++/++
Type 1	++/++	−/−	++/++	−/−	++/++

− = no precipitation; + = moderate and ++ strong precipitation. Readings taken after 2 hr. at 37° and 18 hr. at 4°. N.D. = not done. ++/++ = 2 hr. reading/18 hr. reading.

Pepsin had an interesting effect in that it appeared, from the preliminary results, to digest the antigen, both in the extract and on the living organism. This suggested that the type-4 precipitating antigen was similar in character to the R antigen of type 28 which is digested by pepsin but not by trypsin (Lancefield & Perlmann, 1952*b*). It was found however, that the type-4 antigen, unlike the R antigen of type 28, could be detected in considerable quantity in the supernatant fluid from a pepsin digest even after 18 hr. incubation at 37°. The action of pepsin on type-4 organisms seemed, therefore, to be simply the complete and rapid release of the antigen into solution.

Type-4 cocci were also rendered completely inagglutinable by anti-T sera after treatment with pepsin for 2 hr. at 37°, thus confirming that the responsible antigen had been removed from the cell surface. Pepsin did not have this effect on the other types tested, namely 1, 2 and 3. The antigen responsible for precipitation in acid extracts of type 4 is thus resistant to digestion with trypsin and pepsin while attached to the cells, although it may become sensitive to digestion by these enzymes after extraction by boiling at low pH; it cannot therefore be typical M antigen.

Absorption experiments

Absorption of sera with the crude T fraction completely removed the antibody responsible for precipitation with both Lancefield and Pakula extracts

Table 2. *The absorption of antisera with type-4 T fractions*

Antisera type	Absorbed with T fraction of type	Lancefield extracts of type						Pakula extracts of type	
		4	24	26	29	46	1	1	4
4	—*	++/++	−/−	−/−	−/−	tr/tr	..	..	++++/++++
4	4	−/−	−/−	−/−	−/−	−/−	..	..	−/−
4	1	++/++	−/−	−/−	−/−	tr/tr	..	..	++++/++++
4	2	++/++	−/−	−/−	−/−	tr/tr	..	..	++++/++++
24	—	−/−	++/+++	−/−	−/−	−/−	..	..	−/−
24	4	−/−	++/+++	−/−	−/−	−/−	..	..	−/−
26	—	−/−	−/−	+++/+++	−/−	−/−	..	..	−/−
26	4	−/−	−/−	+++/+++	−/−	−/−	..	..	−/−
29	—	−/−	−/−	−/−	++/++	−/−	..	..	−/−
29	4	−/−	−/−	−/−	++/++	−/−	..	..	−/−
46	—	+/+	−/−	−/−	−/−	++/++	..	..	+++/+++
46	4	−/−	−/−	−/−	−/−	++/++	..	..	−/−
1	—	−/−	..	..	..	..	++/++	++/+++	..
1	4	−/−	..	..	..	..	++/++	++/+++	..
1	1	−/−	..	..	..	..	++/++	−/−	..

* Not absorbed. .. = not tested. Other conventions as in Table 1.

and for agglutination of type-4 cocci (Table 2). Similar doses of type-4 T fraction did not remove the specific precipitating antibody (M antibody) from sera of types 24, 26, 29 and 46 or 1. Nor did a similar dose of T fraction from a type-1 strain remove the precipitating M antibody from a type 1 anti-M serum, although it removed the T antibody also present in that serum.

The fraction prepared from the Lancefield extract of type 4, when used for absorption, not only decreased the ability of the serum to precipitate with type-4 Lancefield extracts but also decreased its agglutination titre from 1/640 to 1/40 (Table 3; agglutination was used to estimate antibody in this experiment for technical reasons). It was not possible, by increasing the absorbing dose, to remove the type-4 agglutinin completely. This may indicate that type 4 has two trypsin-resistant antigens, one of which is extracted by hot acid while the other, like a true T antigen, is destroyed.

Table 3. *The absorption of antisera with type-4 'M' fraction*

Antisera type	Absorbed with 'M' fraction of type	Precipitin reaction of Lancefield extracts of type		Agglutination titre using trypsin-treated cocci of type	
		4	6	4	6
4	−*	++/++	−/−	1/640	N.D.
4	4	−/−	−/−	1/40	N.D.
4	6	++/++	−/−	1/640	N.D.
6	−*	−/−	++/++	..	1/1280
6	4	−/−	++/++	..	1/1280
6	6	−/−	−/−	..	1/1280

Conventions as in Table 1.

Preparation of type 4 anti-T serum

An antiserum was prepared against type-4 streptococci which had been digested with trypsin. An antiserum made in this way to a satisfactory M type, such as 3, 5 or 12, contains no demonstrable M antibody; the type-4 antiserum, however, gave as good precipitin reactions with Lancefield extracts as one prepared against organisms not treated with trypsin.

Preparation of antiserum against freshly isolated strains of type 4

Type-4 strains are isolated from cases of acute illness and one might expect that at some stage or under certain conditions they possess a true M antigen. An attempt was therefore made to produce anti-M sera by the use of freshly isolated strains. Four different vaccines were used for preparing these antisera.

(1) Prepared by the usual method for anti-M sera described under methods, using strains R54.2604 and R55.17.

(2) Prepared by a modification of the method described by Conroy & Updyke (1954) using mechanically disrupted cocci of strains R54.2692 and R54.2707. The cocci were washed, suspended in saline and shaken in a tissue disintegrator (Mickle), until they became Gram-negative and partially disinte-

grated. The suspension was centrifuged at 4000 rev./min. for 30 min., and the deposit was then resuspended in saline and heated at 56°.

(3) A heat-killed unwashed broth culture of strain R55.17.

(4) A culture of strain R54.2692 grown in human blood and killed with formalin.

The six antisera prepared by these methods were tested for precipitins and bactericidal antibodies. All the antisera prepared by these methods, with the exception of that made by method 4, contained precipitins, but in each case these could be shown to be directed to the T antigen. Although three out of four of the strains grew well in fresh heparinized human blood, which in itself suggested the presence of an M antigen, none of the antisera prepared increased the bactericidal activity of blood.

The 'B' antigen present in type-4 strains

It was noticed that extracts of all the freshly isolated type-4 strains tested gave a precipitin reaction with a serum (R432) prepared against the provisional type B3264. The antigen responsible for this reaction, provisionally referred to as the 'B' antigen, is, like the M antigen, best extracted with 0·2N-HCl at 100° from cultures grown at 37° in Todd–Hewitt broth. It can also be extracted by heating at 100° for 10 min. at alkaline or neutral pH values or by autoclaving at 10 lb./sq.in. for 10 min. in pH 7·8 buffer; but not with acid at 50°. The antigen is sensitive to digestion by trypsin or pepsin. It appears to be lost or changed when a strain is subjected to repeated passage through mice.

The main interest of the B antigen is that it is found almost exclusively among types for which satisfactory anti-M sera are difficult to prepare. Lancefield extracts of 332 freshly-isolated strains were tested for precipitation against an antiserum containing B antibody. The 55 extracts that reacted with the anti-B serum were from strains with various agglutination patterns but the only strains whose extracts precipitated with other available sera were of types 2, 4 and 28. The type-4 and -28 sera were certainly not anti-M and the type-2 serum, although it contained antibody to the M antigen, also contained antibody to T. Maxted (1953) has shown that the type-2 T antigen is not typical in its sensitivity to heat so that one cannot be certain whether this reaction was due to M or T. The types that did not precipitate with B antibody were 1, 3, 5, 6, 12, 14, 18, 19, 30.

Among the collection of strains maintained in this laboratory the B antigen has been identified among representatives of types 2, 4, 8, 9, 11, 13, 22, 25, 27, 28, but not all the strains of a particular type possess it. Confusion can result when antisera are prepared against vaccine strains containing a B antigen but are tested against strains lacking it. We have often noted that new antisera for the 'difficult' types appear fully absorbed when tested against the standard set of strains used for preparing M antisera, but give numerous cross-reactions when used for the routine typing of freshly-isolated strains; the strains of the standard set have mostly had numerous mouse passages.

The presence of the B antigen in a strain seems to make it less suitable as a vaccine strain for the production of M antiserum. Even apart from the extra absorption involved, it has been noticed, particularly in the case of type 9, that stronger anti-M serum is produced against a strain that does not possess the B antigen than against others that do.

DISCUSSION

A close relationship exists between M-antigen production and the ability of the strain to resist phagocytosis (Wiley & Wilson, 1956). If it be true that the M antigen is needed for a strain to be virulent, one would expect that strains of type 4 isolated from cases of acute streptococcal disease would not differ in this respect from other types. Yet it has so far proved impossible to demonstrate unequivocally the production of an anti-M serum. This might be due simply to an unfortunate selection of vaccine strains, but there are two other possible explanations. The M antigen, although present in the organism, may be masked by another antigen to which the antibody response is stronger. For example, with type 28, antiserum containing the R antibody is very easily produced; it was only after repeated mouse-passage of a strain that Lancefield was able to produce an antiserum which appears to have the properties of a true anti-M serum (Lancefield & Perlmann, 1952*b*). In the case of type 4, either the type-4 T antigen or the B antigen might be responsible for masking.

Alternatively, the M antigen may be readily lost from the type-4 organism on isolation and passage through laboratory media as happens with some strains (Wormald, 1956).

Against all this, however, there is the possibility that type-4 strains do not possess an M antigen similar to that of other types and that their virulence depends on some other factor. It is curious that several freshly isolated type-4 strains have been found to grow well in human blood—a capacity that characterizes M-containing strains—and yet these strains failed to stimulate the production of M antibodies in rabbits.

Whatever the explanation of these observations, it seems clear that the antigen responsible for the precipitation reaction with our currently available type-4 antisera is not due to an M antigen. This finding, with the comparable observations on type-2 streptococci, emphasizes the necessity of making extensive verification tests before accepting such precipitation reactions as indicating the presence of M antigen.

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The Effect of the Medium on the Cultivation of *Mycobacterium balnei* at 37°

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SUMMARY: *Mycobacterium balnei* grows best at temperatures about 32°. Growth occurs at 37° only on certain media. The organism is more susceptible at 37° than at 32° to the inhibitory effects of oleic acid added to a simple defined medium. This appears to explain the failure of more complex media to support growth at the higher temperature.

Mycobacterium ulcerans, described in 1948 by MacCallum, Tolhurst, Buckle & Sissons, was peculiar among the mycobacteria in the limited temperature range permitting growth and especially for its failure to grow in primary culture at 37°. This observation led directly to the primary cultivation of *M. balnei* by Nordén & Linell (1951), since Lowenstein–Jensen medium inoculated with material from human lesions gave growth when incubated at 31° but not when incubated at 37° (see also Linell & Nordén, 1954.)

Subcultivation of *Mycobacterium ulcerans* on a variety of media has so far failed to give sustained growth at 37°. Subcultivation of *M. balnei* is successful at 37° under certain conditions. Thus Nordén (personal communication, 1955) using large inocula, obtained growth on Lowenstein–Jensen medium at 37°, and Leach & Fenner (1954) reported growth at 37° in Dubos medium containing albumin and Tween 80. Yet subcultures of this organism, made when it was received here, yielded no growth on egg-yolk agar when incubated at 37°. The possible effect of the nature of the medium on growth at different temperatures was therefore considered.

In preliminary experiments the addition of activated charcoal to blood agar or to nutrient broth permitted growth at 37°. The significance of this finding was investigated.

METHODS

Liquid media. The basal medium used was that of Proskauer & Beck (PB) as modified by Youmans (1946) except that the concentration of glycerol was decreased to 1% (v/v). This medium contained: asparagine, 5·0 g.; KH_2PO_4, 5·0 g.; K_2SO_4, 0·5 g.; Mg citrate, 1·5 g.; glycerol, 10 ml.; water to 1000 ml. The pH value was adjusted to 7·0 and the medium autoclaved at 15 lb./sq.in. for 15 min., filtered while hot to remove precipitate, and autoclaved again at 15 lb./sq.in. for 15 min. Liquid media were distributed in 5 ml. vol. in $6 \times \frac{3}{4}$ in. test tubes.

Commercial oleic acid was purified by distillation; 0·12 ml. (0·1 g.) was dissolved in 10 ml. 0·05 N-sodium hydroxide, sterilized by Seitz filtration and diluted in PB medium to give from 3 to 100 p.p.m. as required.

Bovine serum albumin Fraction V (Armour) was used in a concentration of 0·5% (w/v).

Activated charcoal (British Drug Houses Ltd.) was used at 0·2 and 0·4% (w/v).

Solid media used were as follows:

(*a*) Nutrient glycerol agar prepared either with PB medium or with Wright's broth containing 1% (v/v) glycerol but not glucose. Both Davis and Difco brands of agar were used.

(*b*) Albumin glycerol agar: medium (*a*) containing 0·5% (w/v) albumin.

(*c*) Charcoal glycerol agar: medium (*a*) containing 0·2% (w/v) charcoal.

(*d*) Blood glycerol agar: medium (*a*) containing 10% (v/v) citrated horse blood.

(*e*) Charcoal blood glycerol agar: medium (*d*) containing 0·2% (w/v) charcoal.

(*f*) Egg-yolk glycerol agar: K_2HPO_4, 2·4 g.; KH_2PO_4, 0·6 g.; agar 4·0 g.; glycerol, 4·0 ml.; water to 300 ml. After autoclaving this solution at 15 lb./sq.in. for 15 min., the medium was cooled to 60° and 100 ml. sterile egg yolk and 4 ml. of a 2% (w/v) solution of malachite green were added. I am indebted to Mr Glen Buckle for this recipe.

(*g*) Charcoal egg-yolk glycerol agar: medium (*f*) containing 0·2% (w/v) charcoal.

(*h*) Lowenstein–Jensen medium.

(*i*) Dorset egg medium.

Glassware was boiled in soapy water, scrubbed, and rinsed thoroughly with tap water, but special methods of treatment to avoid contamination with traces of fatty acids were not used. Test tubes were plugged with cotton wool and sterilized by dry heat. Before incubation the cotton wool was replaced by bark corks or rubber caps.

Organism. Mycobacterium balnei strain 'X' isolated in 1950 by Linell & Nordén (1954) from a typical human lesion was used. I am indebted to Professor Frank Fenner for a subculture. The strain produces a rough variant which was also used.

Inocula. Cultures were prepared in PB medium containing 0·5% (w/v) serum albumin and 0·1% (v/v) Tween 80 and were incubated at 32° for 7 days. Tenfold dilutions were made with separate 1 ml. pipettes for each dilution; the suspensions were pipetted up and down ten times each.

In the experiments illustrated by Table 1 and in experiments with solid media, 0·1 ml. of the appropriate dilution of culture was delivered into each tube of liquid medium or on to the surface of each tube of solid medium.

In the experiments illustrated by Figs. 1–3 the culture was centrifuged, washed once with PB medium and resuspended in this medium. Tenfold dilutions were made in PB medium except for the dilutions designed for incubation and subsequent counting; these were made in each case in the medium being tested.

Counts were made by the method of Miles & Misra (1938) on egg-yolk agar plates incubated at 32° for 9 days. Since dispersion is imperfect even in media containing Tween 80, and clumps of bacteria as well as single organisms are seen on microscopic examination, Fenner, Martin & Pierce (1949) suggested that counts of mycobacteria should refer to 'bacterial units' rather than to individual bacilli. The errors inherent in counting mycobacteria have been fully discussed by Fenner (1951). Although, in the experiments reported below, satisfactory replica counts were obtained, no corrections have been applied and the counts are therefore to be taken as approximate.

Temperatures of incubation. Culture tubes were stood upright in incubators with a range from 31° to 32°, or from 36° to 37°, respectively. The temperatures were recorded on thermographs.

RESULTS

Growth in liquid media at 32° and at 37°

Preliminary experiments showed that successful cultivation of *Mycobacterium balnei* strain 'X' in PB medium with or without added oleic acid depended on the size of the inoculum. Inocula too small to initiate growth in these media were frequently successful at 32° and at 37°, when either albumin or charcoal was present (Table 1). Fig. 1 illustrates the effect of size of inoculum on growth at 32° and at 37° in PB medium to which had been added 5 p.p.m. oleic acid. With an inoculum of *c.* 1·5 million bacterial units, growth failed at both temperatures; with 3·5 million units it succeeded at 32° but failed at 37°; with 15 million units it was successful at both temperatures. Figs. 2 and 3 show the effect on growth at 32° and at 37° respectively, of incorporating albumin or charcoal in the medium. An inoculum of 1·5 million units which failed to grow in the absence of these substances was successful at both temperatures in the presence of albumin or charcoal. In these experiments growth was followed by counts for a period of only 48 hr. The findings were supported by the subsequent absence or development of floccular macroscopic growth. Repeated experiments gave similar results.

Growth on solid media at 32°

Glycerol agar was a poor medium in that a surface inoculum of *c.* 1 million bacterial units was required to initiate growth. The addition of albumin, charcoal or whole blood permitted the growth of very small inocula of the order of 10 bacterial units, but the colonies were small. On egg-yolk glycerol agar the colonies were larger although not more numerous; luxuriant growth was readily obtained and this was the best solid medium at 32°. Lowenstein–Jensen and Dorset egg media gave similar results.

Growth on solid media at 37°

On all the media tested at 37° large inocula were required to initiate growth. On glycerol agar an inoculum of *c.* 10 million bacterial units was necessary. Following the addition of albumin, charcoal or whole blood, growth could be obtained with *c.* 100,000 units and when charcoal was incorporated in blood agar, growth was obtained with *c.* 10,000 units. In marked contrast to its

Table 1. *Effect of size of inoculum on growth in PB medium containing oleic acid, oleic acid and albumin or oleic acid and charcoal*

Inoculum in bacterial units	Temp.	Oleic acid (parts per million)							Oleic + 0·5 % albumin							Oleic + 0·4 % charcoal						
		0	3	6	12	25	50	100	0	3	6	12	25	50	100	0	3	6	12	25	50	100
10^7	32°	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	37°	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10^5	32°	−	−	−	−	−	−	−	+	c	+	+	+	+	−	+	+	+	+	+	+	+
	37°	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	+	+	+	+	+	+
10^3	32°	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
	37°	−	−	−	−	−	−	−	+	c	c	−	−	−	−	+	+	+	+	+	+	+
10	32°	−	−	−	−	−	−	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+
	37°	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	+	−

+ = Growth; − = no growth; c = contaminated.

behaviour at 32°, egg-yolk glycerol agar was no better than glycerol agar at 37° and was not improved by the addition of charcoal. Lowenstein–Jensen and Dorset media gave similar results. The omission of malachite green from the egg media did not improve them. At both 32° and 37°, similar results were obtained with inocula developing from rough or smooth colony types.

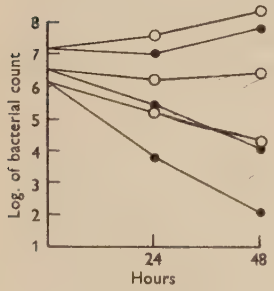

Fig. 1

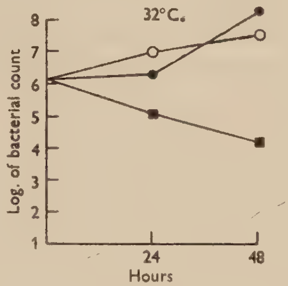

Fig. 2

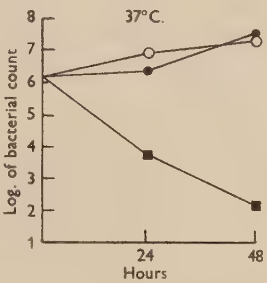

Fig. 3

Fig. 1. Effect of size of inoculum on growth in PB medium containing 5 p.p.m. of oleic acid at 32° (○—○) and 37° (●—●).

Fig. 2. Effect of albumin and charcoal on growth at 32°. PB medium containing 5 p.p.m. of oleic acid alone (■—■), and with addition of 0·5 % albumin (●—●) or of 0·4 % charcoal (○—○).

Fig. 3. Effect of albumin and charcoal on growth at 37°. PB medium containing 5 p.p.m. of oleic acid alone (■—■), and with addition of 0·5 % albumin (●—●) or of 0·4 % charcoal (○—○).

Rate of growth

In PB liquid media containing albumin or charcoal, initial growth at 37° was nearly as rapid as at 32° when inocula greater than a million bacterial units were used. Macroscopic growth was evident in 3–6 days but was less bulky at 37° than at 32°. With small inocula, growth was evident after 6 days at 32°, but was sometimes not apparent at 37° until after 3 weeks.

Cultures from tissues

Cultures were made from lung lesions in mice on the following media: PB + albumin, PB + charcoal, PB + albumin agar, PB + charcoal agar, egg-yolk agar. At 32° heavy growth was obtained on all media. At 37° growth failed on egg-yolk agar. It was successful on all the other media, although much less abundant than at 32°.

DISCUSSION

The inhibitory effect of Long's defined medium (Long & Seibert, 1926) on the growth of *Mycobacterium tuberculosis* and its neutralization by serum or serum albumin was recognized by Boissevain (1940) and by Drea (1942). Boissevain (cited by Drea) found that *M. tuberculosis* was inhibited by 1 p.p.m. oleic acid. Dubos (1947) and Davis & Dubos (1947) showed that this effect was neutralized by serum albumin.

The inhibitory action on various organisms of a substance in agar closely resembling oleic acid has been described, and its neutralization by starch, albumin or charcoal has been demonstrated (Uyei, 1930; Drea, 1940; Ley & Mueller, 1946; Dubos, 1947; Dubos & Middlebrook, 1947; Glass & Kennett, 1939; Pollock, 1947).

Davis (1948) showed that large inocula of *Mycobacterium tuberculosis* could metabolize and remove added oleic acid from a medium not containing albumin. Small inocula unable to grow in the medium itself produced growth in the culture filtrate in which the concentration of oleic acid had been reduced below the inhibitory level.

The observations on *Mycobacterium balnei* recorded above show that the inhibitory effect of oleic acid in PB medium can be neutralized by the addition of albumin or of charcoal or by the use of a large inoculum. They suggest that at least for this organism, a reduction in the temperature of incubation from 37° to 32° may permit growth on certain media because fatty acids in the latter are less inhibitory at 32° than at 37°. Thus the choice of media becomes important in cultures made for diagnosis if they are to be incubated at 37°, particularly when the number of bacteria in a lesion is so small that they are not readily detectable in smears. The absence of growth on egg-yolk media at 37° is particularly striking in contrast to the luxuriant growth obtained on these media at 32°. Egg yolk contains sphingomyelin, lecithin and cholesterol, all of which are capable of binding fatty acids (Dubos, 1948; Kodicek & Worden, 1945), but it is possible that fatty acids remain available in sufficient concentration to cause inhibition of growth at the higher temperature. Reasons for the failure of charcoal to permit growth on egg-yolk agar at 37° have not been investigated.

I am indebted to Mrs J. Keir for skilled technical assistance.

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Development of the Culture of *Streptomyces rimosus* in Submerged Fermentation

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SUMMARY: The development of *Streptomyces rimosus* in submerged culture, in a medium containing starch, glucose, ammonium sulphate, corn-steep liquor and calcium carbonate, takes place in several phases which differ in their morphological and biochemical characteristics. The starting phase is characterized by the logarithmic growth of thick Gram-positive filaments of the primary mycelium. Nucleic acids and respiratory enzymes are being synthesized during this phase and free amino acids and inorganic phosphorus in the medium are being almost completely used up. A considerable amount of pyruvic acid is being produced and accumulated in the medium. No production of oxytetracycline and pigments takes place. In the phase of logarithmic growth the respiration of the culture reaches a maximum value of 500–700 ml. O_2/l./hr.; at this stage growth of the primary mycelium ceases and its hyphae undergo fragmentation. Pyruvic acid, which had accumulated in the medium in the phase of logarithmic growth, is rapidly used up during the fragmentation period. From the fragments of the primary mycelium long Gram-negative filaments of secondary mycelium start growing. Growth in this phase is accompanied neither by any significant increase of the respiration nor by excretion of pyruvic acid; deoxyribonucleic acid (DNA) synthesis is considerably slower than in the phase of logarithmic growth and the total amount of ribonucleic acid (RNA) is decreasing; oxytetracycline and pigments are being produced both by the growing secondary mycelium and in the subsequent stationary phase.

During the growth and development of actinomycetes morphological and physiological changes take place, both in surface cultures (Klieneberger-Nobel, 1947) and in submerged cultures (Carvajal, 1947; Biffi, Boretti, Di Marco & Pennella, 1954; Scotti & Zocchi, 1955; Baldacci, 1956). The development of the culture takes place in roughly two phases: in the first phase long hyphae develop from the spores; these hyphae undergo fragmentation or sporulation; the fragments or spores give rise to the hyphae of the second generation. Similar morphological changes take place also during the growth of *Streptomyces rimosus*, a producer of the antibiotic oxytetracycline (Prokofieva-Belgorskaya, Pestiereva & Rudaya, 1956). A biochemical characterization of the development of this species has so far not been presented; very little information is available especially about the factors which determine the existence and transition of the growth phases. Knowledge of these factors is important not only for our understanding of the physiology of the actinomycetes, but also from the practical standpoint; it enables us to predict the development of the culture and to control it by purposeful combinations of medium composition and other cultivation conditions. Knowledge of these factors which limit growth and determine the transition of one phase into the other is indispensable in experiments concerning the possibility of continuous fermentation.

The aim of the present communication is the description of the development of *Streptomyces rimosus* in submerged culture and a biochemical and morphological characterization of the main phases of its development.

METHODS

Organism. A strain of *Streptomyces rimosus* from the collection of the Antibiotics Research Institute, Roztoky near Prague, was used.

Medium. The organism was cultivated on a modified medium described by Nečásek & Lokvenc (1956) of the following composition: 1% (w/v) technical grade glucose; 1·9% (w/v) potato starch; 1% (w/v) corn-steep liquor; 0·6% (w/v) ammonium sulphate; 0·8% (w/v) calcium carbonate; 0·5% (w/v) sodium chloride; 0·2% (v/v) soybean oil as antifoam. The pH value of the medium before sterilization was adjusted to 6·0, after sterilization (115°, 30 min.) it was pH 6·5–6·8. An analysis of the medium is given in Table 1.

Table 1. *Analysis of the culture medium*

Reducing substances after hydrolysis (as glucose)	35·7–40·5 mg./ml.
Free reducing sugars (as glucose)	7·7–8·9 mg./ml.
Keto acids (as Na pyruvate)	24–30 μg./ml.
Neutral carbonyl compounds (as glyceraldehyde)	190–200 μg./ml.
Total nitrogen	1·82–2·17 mg./ml.
Ammonia nitrogen	1·12–1·33 mg./ml.
α-Amino nitrogen	0·44–0·47 mg./ml.
Total phosphorus	250–300 μg./ml.
Soluble phosphorus	82–84 μg./ml.
Inorganic phosphorus	44–48 μg./ml.
Inorganic soluble phosphorus	24–28 μg./ml.

Note. The neutral carbonyl compounds came from the technical grade glucose used.

Cultivation. Growth of the organism was carried out in 20 l. stainless-steel laboratory fermentation vessels of 10 l. effective capacity. The medium was aerated with 0·5 vol. of air/min. and stirred at 300 rev./min. The rate of oxygen transfer as measured by the sulphite oxidation was 1500 ml. O_2/l./hr. The temperature during growth was maintained at 28°. The vegetative inoculum for the seed-tank was prepared in flasks on a reciprocating shaking machine by a procedure according to Nečásek & Lokvenc (1956). The seed-tank was inoculated by 30–50 ml. of a 24 hr.-inoculum from a flask. The production tank was seeded with 10% (v/v) of the inoculum from the seed-tank; the appropriate inoculation age of the seed was determined by the measurement of the respiration of the culture. A culture with a respiration of 300–500 ml. O_2/l./hr. was usually used for inoculation; this value was reached between 16 and 22 hr. of growth in the seed-tank.

Analytical methods. The production of oxytetracycline was determined by the colorimetric method (Monastero, Means, Grenfell & Hedger, 1951). Total carbohydrate was determined after acid hydrolysis by the method of Bertrand, free reducing sugars also by the method of Bertrand. The α-amino nitrogen was determined according to Schroeder, Kay & Mills (1950), the ammonia nitrogen by steam distillation in the presence of magnesium oxide and titration of the distillate according to Cole & Parks (1949). Total nitrogen after kjeldahlization of the sample was determined colorimetrically with the Nessler reagent (Umbreit, Burris & Stauffer, 1951). Keto acids were determined according to Friedemann & Haugen (1942, 1943). Qualitative differentiation of keto acids was carried out by paper electrophoresis of their 2:4-dinitro-phenylhydrazones (Tauber, 1955). Dry weight of mycelium was determined by centrifugation of 10 ml. culture, resuspension of the sedimented mycelium in water acidified by hydrochloric acid to pH *c.* 1·5 (for dissolution of calcium carbonate), washing with water, twice with acetone and drying at 100° to constant weight. Washing with acetone was necessary in order to remove oil added to the culture as antifoam.

Nucleic acids were extracted from the mycelium by perchloric acid according to Ogur & Rosen (1950). The concentration of nucleic acids was determined spectrophotometrically on the Universalspektrofotometer Zeiss. We noted that the extracts of deoxyribonucleic acid (DNA) showed an absorption maximum at wavelengths somewhat higher than those given by Ogur & Rosen for standard preparations of DNA. Ribonucleic acid (RNA) was determined by measurement of the extinction at 260 mμ., DNA at 274 mμ. directly, in appropriately diluted extracts against solutions of perchloric acid of equal concentration. For verification of the method the concentration of total phosphorus was determined in several samples by the method of Fiske & SubbaRow (1925) and the value of specific extinction/mole of phosphorus, $\epsilon(P)$, was calculated. $\epsilon(P)$ is defined as: $\epsilon(P)=30{\cdot}98E/cl$, where E is the extinction measured, c is the concentration of phosphorus in g./l. and l is the thickness of the liquid layer in cm. For RNA the value $\epsilon(P)=9590$ with a standard deviation of $\pm 6{\cdot}6\,\%$ was found as an average of 17 determinations made between 12 and 72 hr. of growth. This value is within the limits of the variation of the values given in the literature for RNA isolated from various sources. For DNA a value of $\epsilon(P)=5700$ with a standard deviation of $\pm 6{\cdot}1\,\%$ was found, this being somewhat less than the values published for various preparations of DNA (Chargaff & Davidson, 1955, cite values from 6000 to 8000). Determinations of nucleic acids were reproducible within the limits of ± 2–$3\,\%$ in extracts prepared independently from the same starting material.

The respiration of the mycelium was determined both by the direct Warburg method and polarographically. The data were obtained usually by the polarographic method, because it was more rapid than respirometry and very frequent measurements of the respiration were possible. In polarographic measurements we followed the method of Umbreit *et al.* (1951) modified for the measurement of the respiration of suspensions of actinomycetes. Determinations were carried out in a respirometric Warburg vessel without the central well, with a side arm provided with a bored stopper. The vessel, brimful with the diluted suspension of the mycelium saturated with air and brought to 25°, was attached to the rubber stopper carrying the mercury dropping-electrode and a reference antimony anode. The temperature 25° was chosen in order to avoid the necessity of working in a thermostat, which would greatly have prolonged the measurements. The electrodes were polarized by a constant potential difference of 300 mV., branched off from a Kohlrausch bridge. The current passing through the cell, proportional to the instantaneous concentration of oxygen, was measured by a galvanometer of the sensitivity of $7\cdot1 \times 10^{-9}$ A./mm.; the sensitivity was regulated by an Ayrton shunt. The instrument was calibrated by a solution of 0·01 N-KCl saturated with air. Current intensity was read every 30 sec. The contents of the cell, which had to be free from air bubbles, was stirred by a magnetic stirrer between the readings. As the consumption of oxygen in a fully developed culture was too rapid, the native suspensions had to be diluted (up to sixfold) with sterile culture medium. Respiration of the mycelium was calculated from the slope of the straight line corresponding to the depletion of oxygen in the vessel multiplied by the dilution. The results of parallel polarographic determinations from the same material were reproducible to ± 5 %. A series of parallel polarographic and manometric determinations showed a good agreement of the results of both methods.

Values of pH were measured with a glass electrode on a pH meter RFT type 158.

The morphology of the mycelium was followed in microscopic preparations stained by Gram's method. The results given in the following section are based on analyses of the growth of fifteen batches, of which seven were inoculated by 0·3–0·5 % (v/v) of vegetative inoculum from a flask and eight were inoculated by 10 % (v/v) of vegetative inoculum from a seed-tank. Unless the contrary is stated, the characters of all cultures agreed within the limits stated below.

RESULTS

The development of the culture in the seed-tank when inoculated by a small amount (0·3–0·5 %, v/v) of a vegetative inoculum begins by a period lasting several hours, in which it is not possible to detect any changes in the culture by any of the methods mentioned above. The start of the growth can nevertheless be characterized indirectly by analyses of the inoculum used. A 24 hr. culture of the 2nd vegetative stage from flasks had a consumption of oxygen about 50–100 ml. O_2/l./hr., so that with an inoculation ratio of 0·3–0·5 % (v/v) the growth started with an O_2-uptake of 0·15–0·50 ml. O_2/l./hr.

After 12–15 hr. of growth in the seed-tank a considerable increase of the dry weight of the mycelium took place, accompanied by a parallel increase of O_2-uptake. The consumption of oxygen in this phase can be measured accurately and quickly, and is therefore best suited for a quantitative characterization of the development of the culture during this period. Measurements of O_2-uptake at 30 min. intervals showed that O_2-uptake during the first period of this stage increased logarithmically. It can be seen (Fig. 1) that by extrapolation of the experimental values of respiration the duration of the lag-phase may be roughly estimated. This extrapolation gives a value of *c.* 90 min.

The exact length of the lag-phase cannot be determined since criteria for its direct measurement are not experimentally accessible. From Fig. 1 the time required for doubling the O_2-uptake, i.e. a value analogous to the mean generation time, can be determined, being equal to 89 min. at 28°. The dry weight of the mycelium also increased exponentially, but its determination was less exact than that of O_2-uptake. The metabolic coefficient Q_{O_2} therefore remained nearly constant during this period and was equal to 137 ml. O_2/g. dry wt. mycelium/hr., on the average.

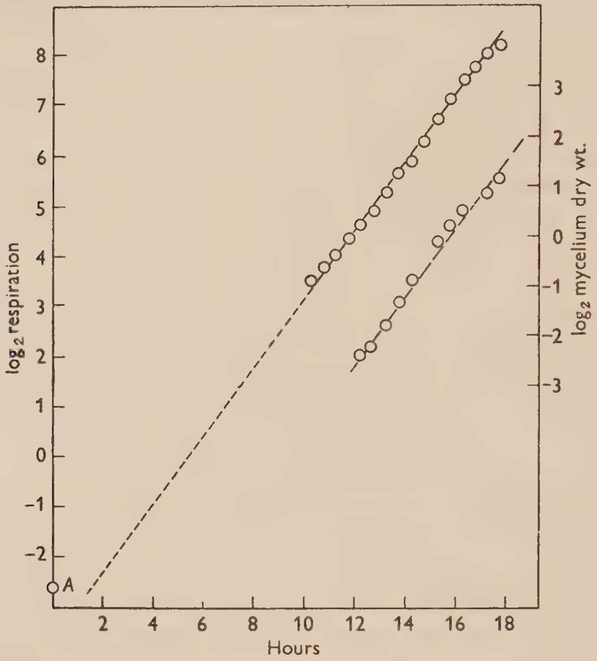

Fig. 1. Stage of logarithmic growth of a submerged culture of *Streptomyces rimosus*. The medium was inoculated with 0·16% (v/v) of a vegetative inoculum. Upper curve: logarithm of O_2-uptake. Lower curve: logarithm of dry wt. mycelium/ml. Point *A* gives the O_2-uptake of the culture at inoculation; this value was calculated from the O_2-uptake of the inoculum.

Nucleic acids were synthesized at a rate parallel to the increase in O_2-uptake and dry wt. mycelium. The amount of RNA synthesized during the stage of logarithmic growth varied in different batches of culture from 240 to 560 μg./ml. The amount of DNA varied between 220 and 270 μg./ml. At the end of the logarithmic growth stage the amount of DNA was always lower than the amount of RNA, although their ratio was not constant. The amount of RNA/unit dry wt. mycelium reached a maximum of 8–12% (w/w) at the end of the logarithmic stage, the amount of DNA then being 5–6%.

Some of the components of the medium were used up almost completely during the period of logarithmic growth. The concentration of α-amino nitrogen decreased from the original 0·44–0·47 mg./ml. to 0·05–0·10 mg./ml. and remained at that level until the end of the cultivation; the concentration of inorganic phosphate decreased from the original 24–28 μg./ml. to 5–6 μg./ml. From the total original amount of ammonia nitrogen (1·12–1·33 mg./ml.) *c.* 0·3–0·4 mg./ml. were used up in this period, so that ammonia is quantitatively about as important a source of nitrogen as amino acids. The amount of total carbohydrate decreased by about 16 mg./ml., whereas the amount of free reducing sugars underwent much less change; reducing sugar was continually being formed from starch by the amylolytic enzymes from the mycelium, so that its concentration remained nearly constant until complete exhaustion of starch.

The increase of the concentration of pyruvate was a very characteristic feature of the stage of logarithmic growth, being completely parallel with the increase in O_2-uptake. The concentration of pyruvate increased to 800–1300 μg./ml. at 8–12 hr. in production tanks, and at 20–25 hr. in the seed-tanks. After reaching this maximum value it decreased again very rapidly (Figs. 2, 4). Consumption of ammonia and formation of pyruvic acid was the cause of the decrease of pH values from 6·8 to 6·3, at which value the culture remained during the rest of the period of cultivation. Microscopically the cultures showed, during the stage of logarithmic growth, a great proportion of long thick Gram-positive filaments and a negligible proportion of disintegrated filaments which were also Gram-positive; very occasionally a few thin Gram-negative filaments may also be observed. In the stage of logarithmic growth the mycelium was white and did not produce oxytetracycline; the first traces of oxytetracycline and pigments began to appear after the maximum concentration of keto acids has been passed.

Logarithmic growth ceased when the O_2-uptake reached 500–700 ml. O_2/l./hr. Growth of the mycelium greatly slowed or even ceased, and O_2-uptake and Q_{O_2} decreased slightly. The rate of utilization of carbohydrate and ammonia-N did not decrease measurably in normal cultures, where this stage was relatively short. When, however, this stage was artificially prolonged, e.g. by interrupted aeration in the previous stage of logarithmic growth, a distinct lag was observed in the curves of carbohydrate and ammonia-N consumption, this lag corresponding to the lag in the growth curve. The concentration of pyruvate decreased from 800–1300 μg./ml. to 20–70 μg./ml. The amount of RNA in this stage decreased, whereas DNA continued to increase, although considerably more slowly than in the stage of logarithmic growth.

Microscopically a complete fragmentation of the thick filaments may be observed in the period after the cessation of logarithmic growth. We therefore designate this stage as that of the fragmentation of primary mycelium.

Under normal conditions of cultivation the stage of fragmentation of primary mycelium lasts *c.* 10 hr. After this period the dry wt. mycelium/ml. begins to increase again and reaches a value of 8–18 mg./ml. during the following 25 hr. The mycelium grown-up in this phase differs profoundly from the primary mycelium growing in the logarithmic phase. The growth of this secondary mycelium was not accompanied by any significant increase of respiration of the culture, so that Q_{O_2} values dropped sharply to 20–50 ml. O_2/g. dry wt. mycelium/hr. No RNA was then being synthesized and its total amount was decreasing. The total amount of DNA continued to increase slowly but not in proportion to the amount of the newly formed secondary mycelium, so that also the amount of DNA/g. dry wt. mycelium decreased slightly (Fig. 3). Usually no pyruvic acid was excreted into the medium. In some anomalous batches of culture the concentration of pyruvate increased slightly (from *c.* 50μg./ml. to ≯200μg./ml.). In these batches also a simultaneous increase of respiration was observed. In these anomalous batches the yield of oxytetracycline was abnormally low (800–1000μg./ml. instead of the usual 1500–1800μg./ml.). Microscopic preparations from this period of cultivation showed a majority of long Gram-negative filaments, later containing Gram-positive inclusions. These thin filaments grow from fragments of the primary mycelium; some of these fragments, however, do not germinate, so that they remain in the medium until the end of cultivation without changing. Occasionally a few long thick filaments of primary mycelium which had not undergone fragmentation were found.

Because of the biochemically and morphologically different character of this mycelium we call it secondary mycelium; in this stage there begins rapid production of oxytetracycline. At the end of the growth of secondary mycelium the culture contains 600–1000μg. oxytetracycline/ml.

The growth of secondary mycelium ceased as soon as ammonia and starch were depleted and the culture entered a stationary phase. Oxytetracycline continued, however, to be synthesized, so that in the stationary phase the maximum of production (1500–1800μg. oxytetracycline/ml.) was reached. Up to 110 hr. of cultivation neither biochemical nor morphological signs of autolysis were observed. The mycelium in the stationary phase showed a gradual fragmentation of the secondary filaments so that towards the end of the cultivation period fragments of the secondary mycelium were most frequently found, although ungerminated fragments of primary mycelium and a few intact filaments of the primary mycelium were also found.

The course of metabolic changes during cultivation is shown in Figs. 2 and 3; the characteristics of various development phases are summarized in Table 2.

The development of the culture in seed-tanks, inoculated with 0·3–0·5% (v/v) of vegetative inoculum did not substantially differ from the development in production tanks with a 10% (v/v) vegetative inoculum. The phase of logarithmic growth with the smaller inoculum lasted, however, *c.* 15–18 hr. longer. This was caused by the fact that different amounts of inoculum reached during their growth the same limiting values, which are probably controlled only by the composition of the medium and the characteristics of the fermentation equipment (Fig. 4).

DISCUSSION

As far as morphological characteristics of the mycelium are concerned, our findings agree roughly with the conclusions of Prokofieva-Belgorskaya *et al.* (1956). Contrary to these authors, however, we found only two types of growing mycelium, which we call primary and secondary, in agreement with

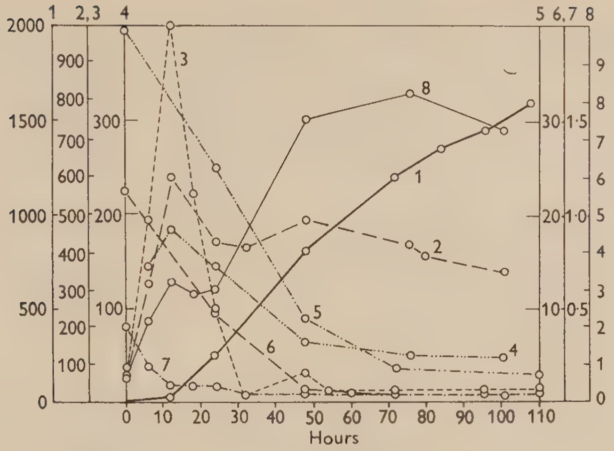

Fig. 2. Metabolic changes in the submerged culture of *Streptomyces rimosus*. The medium was inoculated with 10 % (v/v) of a vegetative inoculum. 1: concentration of oxytetracycline (μg./ml.); 2: O_2-uptake (ml. O_2/l. culture/hr.); 3: concentration of pyruvate (μg. Na pyruvate/ml.); 4: Q_{O_2} (ml. O_2/g. dry wt. mycelium/hr.); 5: concentration of total carbohydrate after hydrolysis (mg. glucose/ml.); 6: concentration of ammonia-nitrogen (mg./ml.); 7: concentration of α-amino-nitrogen (mg./ml.); 8: dry wt. mycelium (mg./ml.).

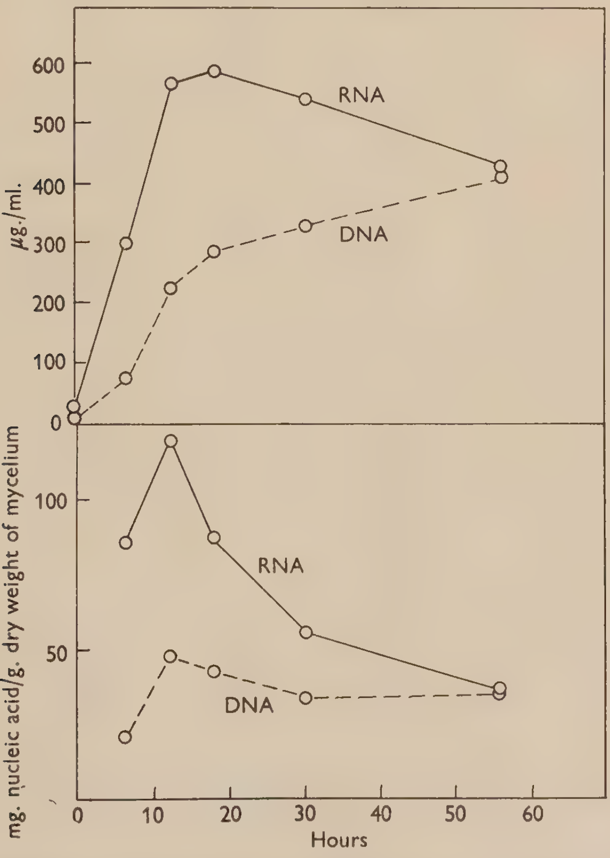

Fig. 3. Metabolism of nucleic acids in *Streptomyces rimosus*. The medium was inoculated with a 10 % (v/v) vegetative inoculum. Upper part: content of nucleic acids/unit of volume; lower part: content of nucleic acids/unit dry wt. mycelium.

Table 2. *Characters of the stages of development of* Streptomyces rimosus *in submerged culture*

	Stage 1. Lag	Stage 2. Growth of primary mycelium	Stage 3. Fragmentation of primary mycelium	Stage 4. Growth of secondary mycelium	Stage 5. Stationary phase
Duration	About 90 min. (only in fermentations with a small amount of inoculum)	10–25 hr. according to amount of inoculum	10hr.	*c.* 25 hr.	The remainder of the cultivation period
Growth of the mycelium	Nil	Up to 4–5 mg./ml.	Ceases	Up to 8–18 mg./ml.	Ceases
Nucleic acids	Nil	Maximum of RNA; rapid synthesis of DNA	RNA decreases; DNA increases slowly	RNA decreases; DNA increases slowly	—
Morphology of the mycelium	Thick Gram-positive filaments	Long thick Gram-positive filaments	Fragmentation of thick filaments; single long thin Gram-negative hyphae	Thin Gram-negative filaments	Fragmentation of thin filaments
Respiration	Not measurable	Exponential increase up to 500–700 ml. O_2/l./hr.	Decreases slightly	Constant or a very slight increase	Decreases slowly 300–400 ml. O_2/l./hr.
Q_{O_2}	Not measurable	Maximum (average: 137 ml. O_2/g. dry wt. mycelium/hr.	Decreases slightly	Decreases sharply 20–50 ml. O_2/g. dry wt./hr.	Decreases slowly
Consumption of nutrients	Nil	Amino-nitrogen and inorganic-phosphorus almost completely used; rapid consumption of sugars and ammonia-nitrogen	Slowed-down consumption of sugars and ammonia-nitrogen	Rapid and complete depletion of sugars and ammonia-nitrogen	—
Pyruvate concentration	24–30 μg./ml.	Rapid increase to 800–1300 μg./ml.	Rapid decrease to 20–70 μg./ml.	Sometimes a slight increase to 200 μg./ml. at the most	40 μg./ml. on the average
Oxytetracycline production	Nil	Nil	Start of production	600–1000 μg./ml.	1500–1800 μg./ml.

older literature. Our primary mycelium corresponds to the first generation of hyphae of Prokofieva-Belgorskaya *et al.*, the secondary mycelium being identical with their second and third generations, which we were not able to differentiate in our experiments. It was to be mentioned that the cultures studied by the above authors produced only *c.* 400μg. oxytetracycline/ml. whereas in our experiments 1500–1800μg./ml. were obtained. The stage of disintegration of primary mycelium was designated as sporulation by Prokofieva-Belgorskaya *et al.* The physiological characteristics of this stage would suggest that this term is not a correct description of what happens. The fragmentation of the filaments is accompanied by only a small decrease of O_2-uptake, and mycelium in this stage, when inoculated at 10% (v/v) into fresh medium grows without any lag. These facts seem incompatible with the definition of spores. We therefore describe the disintegration of the filaments of primary mycelium as fragmentation in the sense of the definition of Krassilnikov (1950).

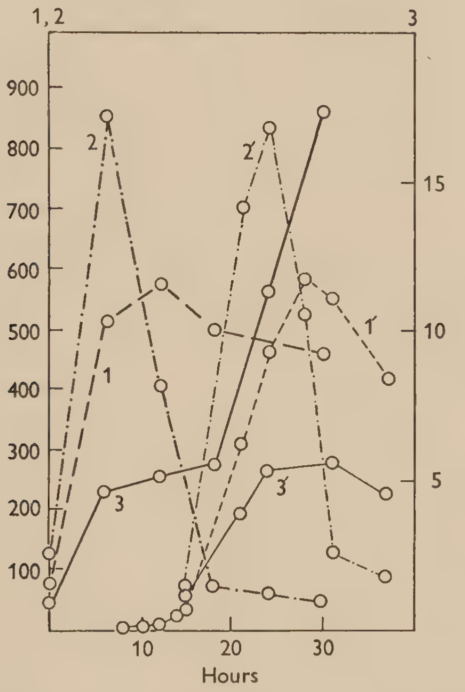

Fig. 4. Comparison of fermentations inoculated with different volumes of vegetative inoculum. 1, 2, 3 = medium inoculated with 10% (v/v) of vegetative culture; 1′, 2′, 3′ = medium inoculated with 0·5% (v/v) of a vegetative culture. 1, 1′ = O_2-uptake (ml. O_2/l. culture/hr.); 2, 2′ = concentration of pyruvate (μg. Na pyruvate/ml.); 3, 3′ = dry wt. mycelium (mg./ml.).

The metabolism of nucleic acids in *Streptomyces aureofaciens* was studied by Biffi *et al.* (1954) and by Guberniev, Ugoleva & Torbochkina (1956*a*), and that of *S. rimosus* by Guberniev, Ugoleva & Torbochkina (1956*b*). The course of nucleic acid metabolism according to our experiments is similar to the results obtained by Guberniev *et al.* (1956*a*) with the species *S. aureofaciens*; we cannot, however, confirm the completely irregular and wide variation of nucleic acid content, recorded by Guberniev *et al.* (1956*b*) for *S. rimosus.*

The growth curve of *Streptomyces rimosus* shows two periods of growth separated by a lag, so that by its form it is reminiscent of the phenomenon of 'diauxie' (Monod, 1942; Hinshelwood, 1946). This behaviour cannot be unequivocally explained on the basis of present experimental material and will be the subject of further study.

The growth of the secondary mycelium, which, contrary to the primary growth, takes place in a medium poor in amino acids, soluble inorganic phosphorus and probably also in oxygen, is limited only by the amount of ammonia and carbohydrates, as it continues until complete depletion of these nutrients.

The practical importance of a differentiation of the types of mycelium lies in the possibility of understanding the relations between the optimum composition of the medium and other fermentation conditions. When, for example, aeration conditions favourable for maximum growth of the primary mycelium are chosen, the concentration of ammonia nitrogen and starch must be correspondingly increased, because too great a part of these nutrients, which are indispensable for the growth of secondary mycelium, will have already been depleted in the stage of logarithmic growth of the primary mycelium. It has been observed several times that a composition of medium which gives satisfactory results in shaking flasks was inadequate in aerated large-scale vessels and addition of ammonium sulphate was then necessary to normalize the production of oxytetracycline.

From the point of view of antibiotic production the indirect dependance between the production of pyruvic acid and biosynthesis of oxytetracycline should be mentioned. These observations, for which some analogies may be found in the literature (Di Marco, Boretti, Julita & Pennella, 1955), may be explained on the basis of Woodward's (1956) hypothesis about the synthesis of tetracycline antibiotics by condensation of several acetyl radicals. The probability of this hypothesis has recently been supported by a direct proof of a similar mechanism in the biosynthesis of griseofulvine, an antibiotic structurally related to the tetracyclines (Birch, Massy-Westropp, Rickards & Smith, 1957). For the biosynthesis of oxytetracycline an excess of acetyl coenzyme A is probably necessary. When the formation of active acetate from pyruvate is hindered by the lack of some necessary coenzyme or intermediate, pyruvate is excreted into the medium and is not utilized for the synthesis of antibiotic. This hypothesis needs, however, a direct experimental verification which would contribute to the solution of the mechanism of the biosynthesis of tetracycline antibiotics.

We wish to thank Docent M. Herold, Director of this Institute, for encouragement and stimulating discussions.

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A Transduction-like Process within a Single Strain of *Pseudomonas aeruginosa*

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SUMMARY: A process of genetic transfer, which could be demonstrated with culture filtrates of a strain of *Pseudomonas aeruginosa*, is described. The evidence indicates that genetic material was transferred in association with a large particle and that the process was similar to Salmonella transduction except that in the present case it occurred spontaneously between mutants of a single strain.

Three processes of genetic exchange have been demonstrated for bacteria. First, there was the process of pneumococcal transformation originally described by Alloway (1932). The active principle was shown to be a deoxyribonucleic acid by Avery, MacLeod & McCarty (1944). Secondly, a sexual process was described by Lederberg & Tatum (1946) for the K12 strain of *Escherichia coli*. Thirdly, Zinder & Lederberg (1952) demonstrated genetic transfer by means of an infectious particle in the genus *Salmonella*.

Loutit (1955) reported a process of apparent genetic transfer which occurred in a strain of *Pseudomonas aeruginosa* during attempts to isolate auxotrophic mutants of that strain. Subsequently, Holloway (1955) reported a sexual recombination in *P. aeruginosa* which occurred between different strains but not within the same strain. On further examination, Holloway (1956) reported that some of his strains were self-fertile, but he found no evidence of a filterable factor concerned either with the transfer process or with sexual compatibility. The present work is concerned with the process described by Loutit (1955), in an attempt to decide whether the process was sexual and if not, to decide whether it was a process of transformation, transduction or in fact a non-specific mutagenic effect.

METHODS

Organisms. The wild-type strain B of *Pseudomonas aeruginosa* was isolated from a wound infection in Brisbane. The auxotrophic mutant organisms were some of those isolated by Loutit (1955) from strain B after ultraviolet irradiation. They were selected by a modification of the single-plate technique of Adelberg & Myers (1953). The mutant strains used predominantly for this work were strains $B17^{ad-}$ (adenineless) and $B21^{l-}$ (leucineless).

Media

Complete medium. Nutrient broth was prepared from Bacto beef extract 3 g. and Bacto peptone 5 g. in 1000 ml. distilled water.

Minimal medium. The medium of Davis & Mingioli (1950) was used, solidified with 1·5% (w/v) washed agar.

Preparation of cuture filtrates. Culture filtrates of the wild-type organism or of either of the mutant strains B 17^{ad-} and B 21^{l-} were obtained by growing these organisms in complete medium at 37° overnight. The cultures were then centrifuged at 3000 rev./min. for 30 min. in an angle-head centrifuge to remove most of the organisms and then the supernatant fluid was filtered through a sterile sintered glass filter (porosity 5/3). At least 2 ml. of every filtrate was transferred aseptically to a sterile bottle and incubated at 37° to test for the absence of living organisms.

Detection of filterable agent in culture filtrates. A just visible suspension of either mutant was prepared in sterile distilled water, and 0·5 ml. of it inoculated into 5 ml. sterile culture filtrate of strain B or the other mutant. At the same time the mutant was inoculated into 5 ml. sterile nutrient broth. The tubes were incubated overnight at 37° and then the organisms were deposited by centrifugation at 2500 rev./min. for 20 min. in an angle-head centrifuge. The organisms were washed twice in 10 ml. sterile distilled water and the organisms from both tubes finally suspended in 0·5 ml. vol. of sterile distilled water.

All the organisms grown in the nutrient broth were spread over the surface of two well-dried minimal agar plates. On the other hand, because of the possible large number of prototrophs in the organisms grown in the culture filtrates, 0·1 ml. of the concentrate from the filtrate was spread over one well-dried minimal agar plate and the remainder of the organisms in the concentrate were diluted 1/10 and then 1/5. Then 0·1 ml. from the 1/10 dilution was spread over the surface of three minimal agar plates and the same procedure was carried out for the 1/50 dilution. The spread plates were placed in the incubator at 37° after the inoculum had dried on the surface and were then incubated and examined at 24 and 48 hr. for prototroph colonies.

Nature of the filterable agent

Action of deoxyribonuclease (DNase). The DNase was obtained from the Mann Research Laboratories, New York, and had been crystallized once. It was tested for activity on thymus deoxyribonucleic acid (DNA), using the viscosimetric methods of assay described by McCarty (1946). For the test on the culture filtrate, a solution containing $100\mu g$. DNase/ml. was prepared in 0·015 M-magnesium sulphate. Three ml. of this DNase solution was added to 12 ml. of a previously prepared culture filtrate of strain B giving a final concentration of $20\mu g$. DNase/ml. in 0·003 M-magnesium sulphate. The tube was incubated for 2 hr. at 37° and then the contents were refiltered through a sterile sintered glass filter (porosity 5/3) and 5 ml. of the filtrate was transferred to a sterile container. Control tubes were prepared by diluting 4 ml. of sterile nutrient broth and 4 ml. of the original culture filtrate with 1 ml. of sterile 0·015 M-$MgSO_4$. All the tubes were then inoculated with 0·5 ml. of a suspension of strain B 21^{l-} in sterile distilled water. The tubes were incubated overnight at 37° and the mutant cultures tested for the presence of prototrophs as described above.

Filtration through Gradocol membranes. A 20 ml. sample of a culture filtrate of strain B was prepared and 10 ml. filtered through each of two sterile Gradocol membranes. The membranes used had average pore diameters (A.P.D.) of 160 and 370 mμ., respectively. Five ml. samples of the filtrates were tested for the presence of a filterable agent after inoculation with strain $B21^{l-}$. At least 2 ml. samples of these filtrates were used to check the sterility of the filtrate.

RESULTS

Detection of a filterable agent

Filtrates from the sintered funnels were always found to be sterile. If either mutant $B17^{ad-}$ or $B21^{l-}$ was inoculated and grown in such culture filtrates of strain B and the mutant organisms were subsequently inoculated on to minimal agar plates, numerous small colonies appeared after 24 hr. incubation at 37°. These colonies were similar to the colonies of the wild-type organism (strain B). A similar result was obtained when one mutant was grown in the culture filtrates from other mutants. In contrast to this, virtually no colonies were seen on any of the control plates inoculated with all the organisms from 5 ml. of broth culture. An occasional colony was seen only once in several experiments. In several experiments, the mutants $B17^{ad-}$ and $B21^{l-}$ were grown in filtrates prepared from strains $B17^{ad-}$ and $B21^{l-}$ respectively. In these experiments no prototrophs were detected.

When quantitative estimations were made of the number of prototrophs great variation was found in the numbers of prototrophs in different culture filtrates. The numbers varied between 500 and 30,000/ml. of culture filtrate. The variation was not so great in replicate tests on the same culture filtrates but there was no correlation between the numbers. Variations from 3500/ml. of culture filtrate to 9900/ml. were demonstrated in the first experiment of Table 1. The figures could be used as an index of the amount of filterable agent only if absorption of the agent occurred at the end of the growth period. If absorption occurred at an earlier stage some of the prototrophs would undoubtedly be progeny of the organisms first treated. Since prediction of the time of absorption would be impossible the quantitative figures could not be used as an index of the amount of filterable agent but only as a qualitative test for the presence of the filterable agent.

Nature of the filterable agent

Action of DNase. The DNase at a concentration of 20 μg./ml. was shown to be an active depolymerase because it brought about a marked decrease in the viscosity of a solution of thymus DNA in 30 min. at 37°. In tests on the culture filtrates the enzyme was used at 20 μg./ml. for 2 hr., although other experiments, using higher concentrations of DNase and longer incubation times, were also carried out. In all these experiments there appeared to be no diminution in the amount of filterable agent as far as could be demonstrated by the number of prototrophs. There was no significant diminution in the number of prototrophs found in culture filtrates treated with DNase compared with that found in the untreated control filtrates. The number of prototrophs was always greater than 500/ml. of culture filtrate. In some experiments the number in the treated filtrate was higher than that in the untreated filtrate while in others the number was lower.

Filtration through Gradocol membranes. The filtrates from the Gradocol membranes were always sterile. When a mutant was inoculated and grown in a culture filtrate from the 160 mμ. A.P.D. filter and the organisms were subsequently plated on minimal agar no prototrophic colonies appeared; but when inoculated and grown in the filtrate from the 370 mμ. A.P.D. filter prototrophic colonies appeared in large numbers. The results of two experiments have been set out in Table 1.

Table 1. *Passage of filterable agent through Gradocol membranes demonstrated by the formation of prototrophs during growth of* Pseudomonas aeruginosa *strain B*21^{1-} *in various culture filtrates of* P. aeruginosa *strain B.*

Growth medium	No. of prototrophs/ml. of culture Expt. 1	 Expt. 2
1. Nutrient broth control	0	0
2. Strain B filtrate from porosity 5/3 sintered glass filter	3500	770
3. Filtrate 2 refiltered through a 160 mμ. A.P.D. Gradocol filter	0	0
4. Filtrate 2 refiltered through a 370 mμ. A.P.D. Gradocol filter	9900	1050

The results of these two experiments demonstrated the variability of the numbers of prototrophs found in different culture filtrates, as well as showing that the filterable agent was unable to pass through the 160 mμ. A.P.D. filter but passed through the 370 mμ. A.P.D. filter.

DISCUSSION

The results described indicate that the process described by Loutit (1955) within the strain B of *Pseudomonas aeruginosa* was not due to a sexual mechanism. There was no difficulty in demonstrating a filterable agent in sterile culture filtrates obtained from large surface area sintered glass filters (porosity 5/3). The U-tube technique described by Davis (1950) was not necessary.

The possibility that the agent was not a genetic transfer agent but was in fact a non-specific mutagenic agent was considered and then discarded since no prototrophs were demonstrated when mutants were grown in their own culture filtrates.

The results indicate that the process is a genetic transfer which is very closely allied to transduction demonstrated for the genus *Salmonella*. The filterable agent was not affected by deoxyribonuclease and seemed to be a relatively large particle. While Stocker, Zinder & Lederberg (1953) stated that the genetic material transferred was probably the same in transformation and transduction, there does seem some point in distinguishing between these two processes, which have a different vector. In the present work it was certainly of interest to know whether some organisms naturally liberated deoxyribonucleic acid which was then taken up by other organisms and incorporated in them, or whether they liberated a particle which was capable of infecting other organisms.

Having established that there was a particle concerned it would be mere speculation at present to discuss the nature of the liberated particle. The only information available concerns the size of the particle; the results suggest that the particle might be as large as 100 mμ. in diameter. The phenomenon does seem to be sufficiently similar to Salmonella transduction to warrant the name of a transduction-like process and does provide additional interest in that transduction occurs spontaneously between organisms differing only in a single genetic characteristic. Zinder & Lederberg (1952) demonstrated that the filterable agent obtained from *Salmonella typhimurium* strain LT-7 or different mutants of that strain could transduce other mutants of the same strain. However, the filterable agent for their work was not produced spontaneously but by an initial chemical treatment and inoculation of the filtrate obtained into broth containing the same strain of organisms. In the present work there appears to be a spontaneous liberation of the infectious particles in sufficient numbers not to require a lytic agent.

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Poliovirus Mutants with Altered Responses to Cystine

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SUMMARY: Three strains of poliovirus (Akron, antigenic type 1; Brooks, type 2; Mabie, type 3), which require exogenously supplied cystine (or a cystine-substitute) for optimal cytopathogenic action on monkey kidney tissue cultures, were serially passaged ten times in cystine-deficient monkey kidney tissue cultures in tubes. From various passage levels in this system were isolated three mutants of Akron, four of Brooks and two of Mabie. These nine mutants are designated the *cystine-response* mutants and are distinguishable from their ancestral viruses used to initiate the passage series by being relatively cystine-non-requiring. Two criteria suffice to distinguish the mutants of any strain from each other: (1) the degree of loss of requirement for cystine, and (2) the degree of inhibition by relatively high concentrations of cystine. The compositions of the virus populations in most of the passage levels were determined, and from these determinations the patterns of emergence of the mutant viruses were delineated.

Several poliovirus mutants which are classifiable on mammalian cells *in vitro* have been obtained. Dulbecco & Vogt (1955) reported an *r* (*rapid*) mutant of the Brunhilde strain (antigenic type 1). This *r* mutant was originally characterized by its relatively large plaques on monkey kidney tissue cultures in a medium containing embryo extract but has since been renamed the *d* (*delayed*) mutant because it can be more readily classified on the basis of its characteristic delay on monkey kidney cells maintained in a relatively acid medium (Vogt, Dulbecco & Wenner, 1957). Several independent occurrences of this type of mutant in other strains of poliovirus have been reported (Sabin, 1956; Vogt *et al.* 1957). A heat-resistant variant of the MEF1 (type 2) strain of poliovirus was obtained by Stanley, Dorman, Ponsford & Larkin (1956) and heat-resistant mutants (t_1 and t_1t_2) of the Brunhilde strain were reported by Dulbecco (1956). Slow Mahoney (Dubes, 1956*a*) is an *m* (*minute*) mutant of the Mahoney strain (type 1) and can be readily classified on the basis of its tiny plaques on monkey kidney tissue cultures. Cold-adapted genetic variants (Dubes & Chapin, 1956) of strains Akron (type 1), Brooks (type 2) and Mabie (type 3) are characterized by their relatively high rate of propagation on monkey kidney tissue cultures maintained at 30°. Genetic variants further adapted to the HeLa cell system have also been obtained (Dubes & Wenner, 1957). Recently, Takemori *et al.* (1957) reported Mahoney and MEF1 mutants which are resistant to the substance(s) which can be found in normal adult bovine sera and which inhibits the 'wild-type' Mahoney and MEF1 viruses.

The demonstration that three strains (Akron, Brooks, Mabie) of poliovirus require exogenously supplied cystine or a cystine-substitute for optimal cytopathogenic action on monkey kidney tissue cultures (Dubes, 1956*b*) and the general success of the technique of passage under conditions favouring the selection of mutants suggested the possibility of obtaining cystine-non-requiring mutants through passage of polioviruses on cystine-deficient monkey kidney tissue cultures. The emergence and characterization of relatively cystine-non-requiring mutants obtained through such selective passages are the subject of this paper. Part of this work has been reported in abstract form (Dubes & Chapin, 1957). The relative virulence in monkeys of some of these mutants has been studied and will be reported elsewhere (Dubes & Wenner, 1957), with a brief summary of their characterization on cells *in vitro*.

METHODS

Poliovirus strains. Akron purified pool no. 1 (PP1), Brooks PP1 and Mabie PP1a, all of which are cystine-requiring strains, were chosen for passage and study. The formation of these purified pools has already been described (Dubes, 1956*a*).

Monkey kidney tissue cultures. Kidney cells from rhesus (*Macaca mulatta*) or cynomolgus (*M. irus*) monkeys were dispersed with trypsin and grown on medium D (Younger, 1954) for 6 days at 35–37° in 150 × 16 mm. test tubes, 60 mm. diameter Petri plates, or 200 ml. screw-cap serum bottles with square cross-section. In a few experiments the growth medium of Melnick (1955) was used in place of medium D. After this growth period the cultures were washed three times with cold phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954), the tube cultures each time with 2·0 ml., the plate cultures each time with 4·25 ml., and the bottle cultures each time with 10 ml. After virus inoculation, the cultures were maintained at 35–37° on 0·90, 6·0 and 10 ml. of a maintenance medium for tubes, plates and bottles, respectively. The maintenance overlay for the Petri plates contained 0·90 % (w/v) washed agar. The maintenance media used were based on Eagle's maintenance medium (E_m) (Dubes, 1956*b*). Medium E_m and an altered E_m (AE_m), which was developed by Miss Joanne Rush for use in this laboratory, were used without cystine, or with cystine at concentrations within the range of 0·050–1·0 mM. Medium AE_m differs from E_m in that it contains Hanks's balanced salt solution (Hanks & Wallace, 1949) in place of the salt solution used by Eagle (1955); the concentration of sodium bicarbonate is, however, kept at 20 mM. All maintenance media were adjusted to a pH value between 6·4 and 6·8 for use in tube and bottle cultures, and to pH 7·0 for use in the plaque method on Petri plates. Tubes and bottles were sealed. Cultures in tubes were rolled after inoculation. Petri plate cultures were kept in a humidified incubator which was continuously flushed with 3 % (v/v) carbon dioxide and 97 % (v/v) air (Dulbecco & Vogt, 1954) and were stained with neutral red 4–8 hr. before examination (Dubes, 1956*a*). In general, the plaque method used was adapted from the original method of Dulbecco & Vogt (1954).

Inoculations with viruses. Viruses were diluted in PBS; 0·10, 0·30 and 1·0 ml. of each appropriate dilution were inoculated into tube, plate and bottle cultures, respectively.

Storage of viruses. All virus stocks were stored in rubber-stoppered ampoules in a dry-ice box.

Passages of viruses on kidney cultures unsupplied with cystine. Samples from Akron PP1, Brooks PP1 and Mabie PP1a were passaged ten times serially in tissue cultures maintained on E_m without cystine. The inoculum for each passage was virus usually at 10^{-2} concentration, equivalent to 10^3 to 10^5 plaque-producing particles inoculated per tube. Between two and six tubes were used for each passage of each strain. Tube cultures were harvested when they exhibited 90–100% cytopathogenic degeneration. The tenth-passage virus of each strain was then passaged three times serially by the plaque method on cells maintained on E_m minus cystine. Each third serial plaque isolate was then inoculated for pool formation into several bottle cultures on the same medium. The pools so obtained were designated Akron PP5, Brooks PP5 and Mabie PP5. The virulence in monkeys of these three viruses has been studied (Dubes & Wenner, 1957).

Genetic purification and pool formation with the other viruses emerging during the passages. From various of the tube-passage levels polioviruses differing from both the PP1's and PP5's were isolated. Purifications and pool formations with these viruses were performed in the same manner as with the PP5's, except that in some cases the purifications by the plaque method were performed on cystine-supplied cells so that plaques of desired appearance might be selected for isolation.

Typing of viruses. The antigenic type of each virus pool was confirmed in neutralization experiments against the three specific poliovirus antisera formed in monkeys (Wenner, Miller, Kamitsuka & Wilson, 1954) and against normal monkey serum as a control.

Symbols for the mutant viruses. To avoid unwieldy descriptions when referring to the various mutants the designations and symbols defined in Table 1 have been used. These symbols do not imply that the phenotypic differences between the various mutants are due to single genetic differences. Akron PP1, Brooks PP1 and Mabie PP1a, the cystine-requiring viruses from which the mutants were derived, are referred to as wild-type ($+^{cr}$) viruses in regard to cystine response. Akron PP5 and Mabie PP5 are cr^i viruses, whereas Brooks PP5 is *cr* virus. The criterion of degree of inhibition by cystine (Table 1) is based on the 'turbidity' of plaques produced on cells supplied with a relatively high concentration (0·20–1·0 mM) of L-cystine. 'Turbid' plaques are plaques which contain a relatively large fraction of neutral red-stainable cells.

Environmental factors affecting the cystine response. The manifestion of a requirement for cystine by the cystine-requiring polioviruses can be modified by varying the temperature during viral action or by establishing the kidney cultures on a different growth medium. For these viruses a lowering of the temperature from 37° increases the relative plating efficiency on cells not supplied with cystine. Substituting the growth medium of Melnick (1955) for medium D has a similar effect. These modifications may operate through altering the size of the intracellular pool of cystine or 'cystine-activity', the influence of the composition of the growth medium being on the size of this pool at the time of inoculation and the influence of temperature being on the rate of metabolic change of this pool after inoculation.

Table 1. *Designations and symbols for wild-type and* cystine-response (cr) *mutant polioviruses*

++ = clear requirement for, or strong inhibition by, cystine. + = slight requirement for, or slight inhibition by, cystine. 0 = no demonstrated requirement for, or no demonstrated inhibition by, cystine.

		Response to cystine	
Virus designation	Virus symbol	Requirement for	Inhibition by
Wild-type	$+^{cr}$	++	0
Cystine-response—partial	cr^{p}	+	0
Cystine-response	*cr*	0	0
Cystine-response—slightly inhibited	cr^{si}	0	+
Cystine-response—inhibited	cr^{i}	0	++

RESULTS

Akron cr *mutants*

Early tests demonstrated clearly that Akron PP5 (cr^{i}) differs from its progenitor PP1 ($+^{cr}$) virus in response to cystine. The Akron cr^{i} mutant shows no requirement for exogenously supplied cystine under the conditions employed; moreover, it produces 'turbid' plaques on monkey kidney cells under an agar layer which contains a relatively high concentration (0·20 to 1·0 mM) of L-cystine.

Platings of the various tube-passage virus populations leading to the isolation of the cr^{i} mutant were made on cells unsupplied and on cells supplied with cystine. In these experiments the $+^{cr}$ and cr^{i} viruses were similarly plated so that comparisons could be made with the viruses at different passage levels using three criteria: (1) plating efficiency without cystine relative to efficiency with cystine; (2) plaque size with and without cystine; and (3) plaque appearance with and without cystine. The results obtained from these comparisons indicated that two other viruses, the *cr* and cr^{si} mutants, which are different from each other and from both the $+^{cr}$ and the cr^{i} viruses, were also obtained in the series of passages on cystine-deficient cells in tubes. Purified pools of the *cr* and cr^{si} mutants were prepared from isolates obtained from tube-passage levels 2 and 5, respectively. The *cr* mutant may be characterized as cystine-non-requiring and cystine-uninhibited and the cr^{si} mutant as cystine-non-requiring and slightly cystine-inhibited. The data from a typical experiment showing the differentiation of these three Akron *cystine-response* mutants and of wild-type Akron are presented in Table 2. In this experiment the cells had been grown on medium D and plaques developed for 4·0 days in a continuously flushed incubator at 36·6°. The difference between the $+^{cr}$ and cr^{i} viruses is illustrated by Pl. 1, fig. 1.

The proportions of the $+^{cr}$, *cr*, cr^{si} and cr^{i} Akron polioviruses in the various tube-passage levels were estimated by using the three criteria mentioned above (relative plating efficiency, plaque size and plaque appearance) and by characterization of the viruses in some plaque isolates from some passage levels by the same criteria. Such classifications of the viruses in the passage levels gave the pattern of emergence of these three Akron mutants presented in Fig. 1.

Table 2. *The differentiating characteristics of the wild-type and* cystine-response *mutant Akron, Brooks and Mabie polioviruses on monkey kidney cells* in vitro

		Differentiating characteristics			
		Mean plaque diameter (mm.)		Plaque appearance	
Poliovirus	RPE (%)	With cystine*	Without cystine†	With cystine*	Without cystine†
Akron $+^{cr}$	0·14	3·7	1·6	Clear	Clear
Akron cr	71	4·4	4·3	Clear	Clear
Akron cr^{si}	190	2·5	6·0	Slightly turbid	Clear
Akron cr^{i}	130	3·9	5·6	Turbid	Clear
Brooks $+^{cr}$	0·069	7·1	2·9	Clear	Clear
Brooks cr^{p}	57	7·8	4·0	Clear	Clear
Brooks cr^{i}	120	5·4	3·4	Turbid	Slightly turbid
Brooks cr^{si}	110	5·7	5·9	Slightly turbid	Clear
Brooks cr	200	6·4	6·0	Clear	Clear
Mabie $+^{cr}$	2·1	7·0	1·2	Clear	Clear
Mabie cr^{si}	120	4·8	4·5	Slightly turbid	Clear
Mabie cr^{i}	260	3·5	4·8	Turbid	Clear

* Medium AEm with L-cystine at 1·0 mM for Akron and Brooks and at 0·50 mM for Mabie.

† Medium AEm without cystine. RPE (relative plating efficiency) is the plating efficiency without cystine relative to the efficiency with cystine. The data are from 3 experiments, 1 for each strain. See Table 1 for an explanation of the symbols for the viruses.

Brooks cr *mutants*

The general procedure followed with Akron was also used with Brooks. The virus in Brooks PP 5 produces clear plaques in the presence of high concentrations of cystine, in contrast to the turbid plaques produced by Akron PP 5, and is thus designated *cr*. This *cr* mutant may be differentiated from the original Brooks $+^{cr}$ virus on the basis of its lack of requirement for exogenously supplied cystine. Comparisons of the tube-passage virus populations with the $+^{cr}$ and *cr* viruses indicated that three other mutants, the cr^{p}, cr^{i} and cr^{si} mutants, also emerged during the series of tube passages. Purified pools of the cr^{p}, cr^{i} and cr^{si} mutants were prepared from isolates obtained from tube-passage levels 1, 2 and 4, respectively. In Table 2 data from a typical experiment showing the distinguishing characteristics of these three mutants and the $+^{cr}$ and *cr* viruses are presented. In this experiment the cells had been grown on medium D and the plaques developed for 4·0 days in a continuously flushed incubator at 35·8°.

The pattern of emergence of these four Brooks *cystine-response* mutants was determined in the same way as was the pattern for Akron and is shown in Fig. 1.

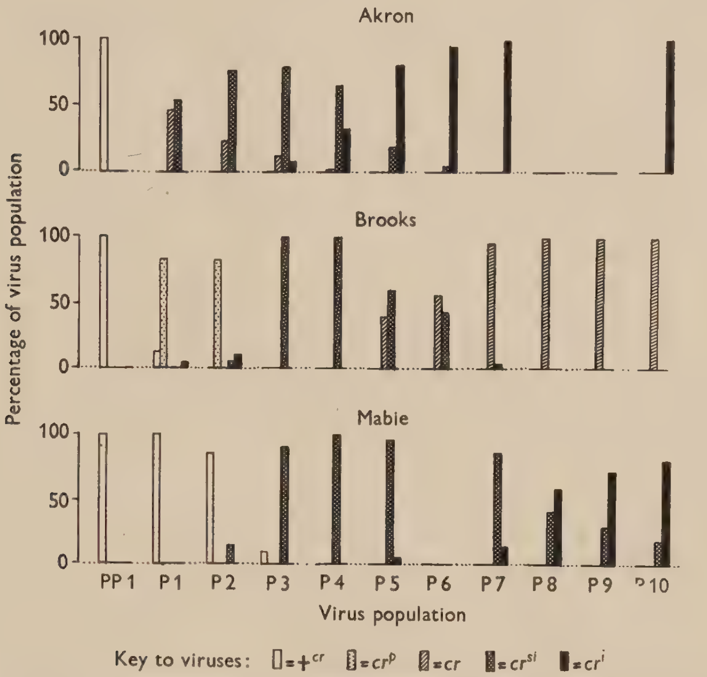

Fig. 1. The patterns of emergence of the *cystine-response* mutants. PP1 (PP1a for Mabie) is the starting pool of $+^{cr}$ virus. Designations P1 through P10 indicate the serial numbers of rapid consecutive passages in tube culture. See Table 1 for an explanation of the symbols for the viruses. Data for P8 and P9 of Akron and for P6 of Mabie were not obtained.

Mabie cr *mutants*

Mabie PP5 contains a virus which has a cystine-response similar to that of the Akron cr^i mutant and is thus called the Mabie cr^i mutant. Another Mabie mutant, the cr^{si} mutant, also emerged during the tube passages. A purified pool of this mutant was prepared from an isolate from tube-passage level 5. Data which show the differentiation of the Mabie cr^{si}, cr^i and $+^{cr}$ viruses are given in Table 2. These data were taken from a typical experiment in which the cells had grown on medium D and the plaques developed for 4·0 days in a continuously flushed incubator at 36·7°. A visual comparison of the $+^{cr}$ and cr^i viruses is afforded by Pl. 1, fig. 2.

The pattern of emergence of these two Mabie *cystine-response* mutants was determined and is shown in Fig. 1.

Tests of progeny viruses in plaques formed by $+^{cr}$ *viruses on cells not supplied with cystine*

Since viruses with altered responses to cystine appeared so very early in the passage series (Fig. 1) experiments were done to see if a single-plaque passage of a $+^{cr}$ virus on cystine-deficient cells would give a virus with an altered cystine-response. Nine, 7 and 3 plaques formed by Akron, Brooks and Mabie $+^{cr}$ viruses, respectively, on cystine-deficient cells were isolated and the cystine-responses of the progeny viruses therein were determined. Three of the Akron plaque isolates were characterized in tests performed in tube cultures, a method previously described (Dubes, 1956*b*). By these tests the three isolates were not unlike their Akron $+^{cr}$ parent. The other 6 Akron isolates were studied more precisely by the plaque method; one was found to be similar to the Akron *cr* virus and the other 5 were found to be intermediate between $+^{cr}$ and *cr*. Two of the latter group of 5 isolates were further resolved by characterization of plaque subisolates. From one of these a virus of phenotype intermediate between $+^{cr}$ and *cr* and a virus somewhat similar to but probably different from cr^{si} were isolated. From the other isolate the two subisolates tested proved to be $+^{cr}$. Three of the 7 Brooks isolates were studied in tests in tube cultures and not found to be different from Brooks $+^{cr}$ virus. The other 4 Brooks isolates were studied by the plaque method; 2 were found to be like the Brooks cr^{p} virus and 2 were found to be intermediate between the $+^{cr}$ and cr^{p} viruses. From one of these 'intermediate' isolates 5 subisolates were obtained. Three of these 5 subisolates proved to be like the original 'intermediate' isolate, one was found to be similar to the Brooks cr^{i} virus, and one was $+^{cr}$ virus. The three Mabie isolates were tested in tube cultures and not found to differ from their progenitor virus, Mabie $+^{cr}$. No tests of Mabie isolates by the plaque method were made since the results of these tests in tube cultures and the finding that tube-passage level 1 contained $+^{cr}$ virus (Fig. 1) show that *cystine-response* mutants of Mabie do not *necessarily* appear after propagation of Mabie $+^{cr}$ virus on cystine-deficient cells.

Thus relatively cystine-non-requiring viruses of strains Akron and Brooks may be obtained by the isolation of plaques formed after the inoculation of the $+^{cr}$ viruses on to cystine-deficient cells. The data also indicate that some of the progeny viruses in these plaques have an unaltered cystine-response.

Tests of progeny viruses in plaques formed by the Akron cr^{i}, *Brooks* cr *and Mabie* cr^{i} *viruses (PP 5's) on cystine-supplied cells*

Plaques formed by Akron cr^{i}, Brooks *cr* and Mabie cr^{i} viruses on cystine-supplied cells were examined for the presence of viruses with cystine-responses different from those of their parents. Such viruses might possibly arise from genetic reversions or simply from the fact that they were synthesized in cystine-supplied cells (cf. Luria, 1953). Seven isolates from typical Akron cr^{i} plaques were tested and found to contain cr^{i} virus. An isolation was also made from an atypical Akron cr^{i} plaque. This plaque was atypical in that it contained a relatively clear sector, i.e. a region in which a relatively low fraction of the cells had concentrated the neutral red. Such atypical sectored plaques are not infrequently found when Akron cr^{i} virus is plated on cells supplied with cystine at 0·20–1·0 mM. From the relatively clear sector of this plaque was isolated a virus unlike cr^{i} but somewhat similar to $+^{cr}$. It differed, however, from $+^{cr}$ in producing slightly turbid instead of clear plaques on cystine-supplied cells and possibly also in having an even lower relative plating efficiency on cells not supplied with cystine. Eight and 6 typical plaques from Brooks *cr* and Mabie cr^i, respectively, were isolated. Each contained virus like its parent.

These findings show that the progeny viruses in typical plaques formed by the cystine-non-requiring Akron cr^i, Brooks *cr* and Mabie cr^i viruses on cystine-supplied cells are like their parental viruses. Thus the nature of these progeny viruses is not determined by the amount of cystine supplied to the cells in which they were synthesized. The cystine-requiring virus which was obtained from the sector of a sectored plaque formed by Akron cr^i virus very probably arose through genetic reversion from cr^i during the propagation of the cr^i virus on the cystine-supplied cells.

DISCUSSION

The patterns of emergence of the relatively cystine-non-requiring viruses during the passages in tube cultures and the isolations of $+^{cr}$ viruses from the progeny viruses in some of the plaques formed by the $+^{cr}$ viruses on cells not supplied with cystine indicate that the variant viruses were obtained through the processes of mutation and selection. The ease of obtaining relatively cystine-non-requiring mutants, of the Akron and Brooks strains especially, is emphasized by the fact that the characterization by the plaque method of the progeny in 6 plaques formed by Akron $+^{cr}$ virus and of 4 plaques formed by Brooks $+^{cr}$ virus on cells not supplied with cystine revealed the presence of at least three different kinds of *cystine-response* mutants of Akron and at least three of Brooks. These results suggest that the formation of most of the plaques produced by Akron $+^{cr}$ and Brooks $+^{cr}$ on cystine-deficient cells under the conditions usually employed herein is in good part due to mutation from $+^{cr}$ to various relatively cystine-non-requiring mutants during the limited propagation of the $+^{cr}$ viruses on the plate cultures.

The isolation from a relatively clear sector of an otherwise turbid plaque formed by Akron cr^i virus on cystine-supplied cells of a virus genetically reverted from cr^i indicates the genetic lability of Akron cr^i virus. It is interesting to note that the cystine-non-requiring characteristic of Akron cr^i virus is apparently completely revertible in one mutational step. The ease of locating the mutant sectors in this system suggests that it may be useful for a study of the process of mutation in polioviruses.

The success of these passages of polioviruses on cystine-deficient monkey kidney tissue cultures in obtaining *cystine-response* mutants and the analogous success in obtaining cold-adapted genetic variants (Dubes & Chapin, 1956) indicate the general usefulness of the technique of passage on cells under abnormal conditions. Thus one kind of host cell may be utilized to obtain different classes of mutant viruses simply by altering the physicochemical environment for the cell-virus system in different ways.

The de-adaptation to the original host cell system (cystine-supplied cells) shown by the Akron and Mabie cr^i viruses is analogous to the de-adaptation to cells at 37° shown by the cold-adapted genetic variants of Akron, Brooks and Mabie (Dubes & Chapin, 1956; Dubes & Wenner, 1957) and reminiscent of the classic de-adaptation to the original animal host observed in animal viruses after serial passage in a different animal host (e.g., Pasteur, Chamberland & Roux, 1884).

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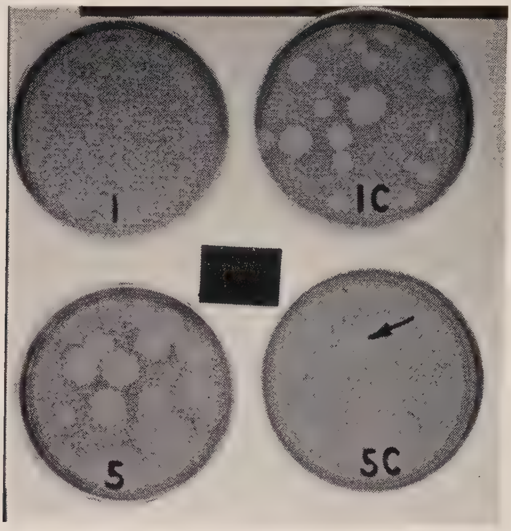

Fig. 1

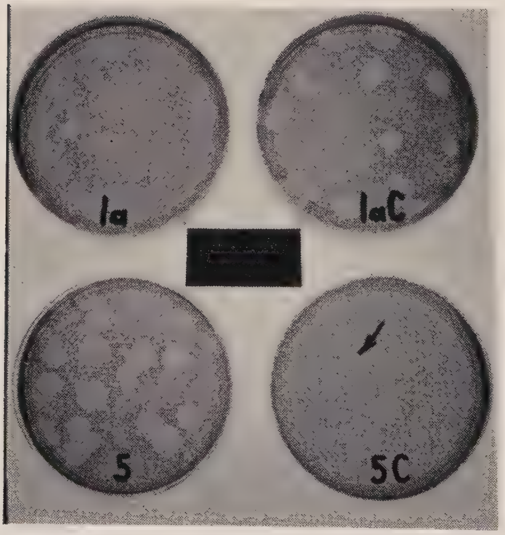

Fig. 2

G. R. Dubes and M. Chapin—Cystine-response mutants of polioviruses. Plate 1

EXPLANATION OF PLATE

Fig. 1. A comparison of plaques formed by Akron $+^{cr}$ virus (PP1) and by Akron cr^{i} virus (PP5) on cystine-supplied monkey kidney cells and on monkey kidney cells not supplied with cystine. The plaques developed on medium D-grown cells for 4·0 days at 35·9°. Plates labelled 1 and 1C each received 0·30 ml. Akron PP1 at 10^{-5} virus concentration and plates 5 and 5C each received 0·30 ml. Akron PP5 at 10^{-4} virus concentration. The maintenance medium for plates 1C and 5C was AEm with L-cystine at 1·0 mM for 1 and 5 was AEm without cystine. No plaques were found on plate 1. The arrow on plate 5C points to a turbid plaque with a diameter of 4·0 mm. Seven turbid plaques were found on plate 5C.

Fig. 2. A comparison of plaques formed by Mabie $+^{cr}$ virus (PP1a) and by Mabie cr^{i} virus (PP5) on cystine-supplied monkey kidney cells and on monkey kidney cells not supplied with cystine. The plaques developed on medium D-grown cells for 4·0 days at 35·9°. Plates labelled 1a and 1aC each received 0·30 ml. Mabie PP1a at $10^{-4\cdot4}$ virus concentration and plates 5 and 5C each received 0·30 ml. Mabie PP5 at $10^{-4\cdot7}$ concentration. The maintenance medium for plates 1aC and 5C was AEm with L-cystine at 1·0 mM and for 1a and 5 was AEm without cystine. Seven small plaques were found on plate 1a. The rather large ill-defined lighter areas on plate 1a are not plaques; they are typical of cystine-deficient monolayers and may be found on control plates uninoculated with virus. Two turbid plaques were found on 5C; one of these with a diameter of 2·5 mm. is indicated by an arrow.

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The Properties of a Star Mutant of Phage T2

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SUMMARY: The properties of a plaque-type variant, or 'star' mutant, of coliphage T2 are investigated. This mutant forms sectored plaques which are composed of approximately equal numbers of the parental star phage particles and of rapid-lysing (r) mutants. The star mutant is shown to be a one-step mutant of wild-type, possessing a mutation at a locus, s_1, that is close to the r_1 locus of phage T2. The r mutants occurring in the star plaques are all double-mutants, containing as well as the s_1 mutation, a mutation at some further r locus. Rapid-lysing mutants isolated from different star plaques possess r mutations at different loci. The high proportion of r mutants occurring in the star plaques is due to selection of r mutants that are formed spontaneously during replication of the star phage. This selection occurs during phage multiplication in *Escherichia coli* that is no longer multiplying exponentially (old bacteria), but not in exponentially growing bacteria. The slow multiplication of star compared to r phage in old cultures of *E. coli* is not due to an inability of the old bacteria to synthesize star phage particles at the same rate as r phage particles, but is caused by two, probably related, factors. First, the star phage has a prolonged latent period in old bacteria, and secondly, the star phage particles that are eventually released when the old cells lyse re-adsorb very slowly to old bacteria in the culture. During multiplication in old bacteria the r phage therefore goes through many more cycles of growth than the s phage, and thus accumulates more rapidly.

Wild-type coliphages of the related strains T2, T4 and T6 form plaques which have a small dark centre surrounded by a pale halo. The most common type of mutation that affects plaque-type in these phages is the so-called r, or rapid-lysing mutation. These r mutations occur with a frequency of approximately one per thousand duplications, and give rise to a distinctive type of plaque that is much clearer than that of the wild-type phage (Hershey, 1946). Another plaque-type variant, which only occurs rarely, was discovered by Hershey (1946) in a stock of T6. The plaques of this variant have the dark centre surrounded by a pale halo that characterizes the wild-type plaques, but surrounding the halo are a number of areas of rapid lysis which give the plaques a characteristic sectored appearance. When these variant plaques are picked and replated one always obtains the same sectored plaques in a mixture with about an equal number of typical r plaques. On picking these secondary sectored plaques one again obtains a mixture of sectored and r plaques, but picking from the secondary r plaques yields pure r stocks. Hershey suggested that the high proportion of r mutants found in these sectored plaques was due to a mutational instability in the parental phage, and he called this variant an 'unstable' mutant.

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Subsequently other 'unstable' mutants were found in coliphage T4 by Doermann & Dissoway (1949) and in phage T2 by Hershey (Visconti, Garen & Symonds, 1953). It was shown with these mutants that the high proportion of *r* mutants found in the sectored plaques was not due to a mutational instability, but was caused by a selective mechanism which allowed the *r* mutants to multiply much faster than the 'unstable' phage under the physiological conditions existing on agar plates during plaque formation. As these mutants do not have a mutational instability the name 'unstable' is inappropriate. We shall call variants whose plaques contain a high proportion of mutant types, star or *s* mutants. More recently other star mutants, all of which form plaques with a somewhat similar morphology, have been reported in coliphage T2 (Baylor, Hurst, Allen & Bertani, 1957; McFall & Stent, 1958), in coliphage T1 (Bresch, 1953; Tessman, 1956), and in salmonella phage (Terada, Kondo & Ogawa, 1956).

In this paper we shall discuss the properties of the star mutant discovered by Hershey (Visconti *et al.* 1953). Two main problems will be investigated: (1) the genetic structure of this phage and of the mutants that are found in the star plaques; (2) the mechanism of selection that is responsible for the large proportion of mutants found in the star plaques. Some preliminary results of this work, carried out in collaboration with Visconti, have already been published (Visconti *et al.* 1953).

METHODS

Bacteria. Escherichia coli strain R2 (Hershey, Dixon & Chase, 1953) was used throughout the experiments.

Phage. All the phage strains were mutants of T2H. The viral mutations used as markers were:

(*a*) *Host-range* (*h*) Hershey & Rotman (1949). The genetic factor *h* was carried in all the phage stocks used in the experiments described in this paper, therefore its presence will not be specifically indicated.

(*b*) *Plaque-type.* The different mutants employed were:

(1) Wild-type (*w*).

(2) Rapid-lysing (*r*). The 3 mutants r_1, r_7, r_{13}, isolated and mapped by Hershey & Rotman (1949), were used as reference markers. The r_7 and r_{13} loci are linked with a frequency of recombination of about 14%; the r_1 locus is unlinked (i.e. the frequency of recombination is $\geqslant 30\%$) both to r_7 and r_{13}.

(3) Minute (*m*). This was also used as a reference marker. The *m* locus is unlinked to r_1, r_7 and r_{13}.

(4) Star (*s*). This was isolated by Hershey (Visconti *et al.* 1953). Photographs of plaques of these mutants are presented in Pl. 1.

Preparation of phage stocks. Because of the large number of *r* mutants normally present in the star plaques, it was not possible to prepare stocks of *s* in the usual way. In order to obtain relatively pure high-titre stocks of *s*, a strain of *Escherichia coli*, N-1, that had been isolated by Nelson (Streisinger, 1956) was employed. On plates seeded with this coli, the *s* mutant formed very small plaques containing about 10^7 phage particles, less than 1 % of which were *r* mutants. When all the phage particles comprising one of these plaques was inoculated directly on to a single plate seeded with coli N-1, confluent lysis occurred. From such plates, stocks of the *s* mutant were obtained that contained less than 0·2 % of *r* mutants and had titres greater than 10^{11}/ml. Stocks of the other mutants were prepared by standard procedures (Adams, 1950).

Growth medium (*broth*). Bacto-peptone 2 % (w/v), Lemco meat extract 0·3 % (w/v), glucose 0·1 % (w/v), 0·1 M-NaCl, 10^{-3} M-$MgSO_4$, pH 7·0.

Phage crosses. Crosses were performed by infecting bacteria suspended in buffer with an equal multiplicity of the two parental types, according to the procedure described by Visconti & Delbrück (1953). In all crosses the average multiplicity of infection with each parent was 5. Burst sizes in different experiments ranged from 80 to 400. Equal yields of both parental types were obtained in the progeny of all the crosses.

Growth conditions. When investigating the growth of the star phage in broth cultures, experiments were performed when the bacteria in the cultures were in two distinct physiological conditions. For convenience we shall term the bacteria in these two physiological conditions either 'young' or 'old'. Young bacteria are those which have been grown, with aeration, to a concentration of 10^7 organisms/ml. from a 10^{-3} dilution of an unaerated overnight culture. Old bacteria are those from an unaerated overnight culture, at a concentration of about 10^9 organisms/ml., and are aerated from the time phage is added. All experiments were performed at 37°.

Phage adsorption. In all growth experiments, adsorption was carried out by adding phage particles to bacteria growing in broth, and after a short time (from $\frac{1}{2}$ to 2 min.) adding antiserum to neutralize the unadsorbed phage particles and so limit the adsorption time. Usually about 2 % of the phage particles were adsorbed in 1 min. either to young bacteria at a concentration of 10^7/ml., or to old bacteria at a concentration of 10^9/ml. All growth experiments were performed under conditions of single infection.

Intracellular development of phage. The average number of phage particles/bacterium that was present in infected cultures at different times during the latent period was determined by adding cyanide to samples of the culture, and assaying the number of phage particles released after lysis (Doermann, 1952). In order to determine the rate of increase in the fraction of infected bacteria in the culture which contained at least one phage particle, a streptomycin technique was used. At various times after infection samples from the infected bacteria (which, like the *Escherichia coli* R2, were sensitive to streptomycin) were removed from the culture and immediately plated on agar plates containing 2000 μg. equivalents streptomycin/ml. and seeded with a streptomycin resistant indicator strain of *E. coli* strain B. At this concentration the streptomycin requires 2–3 min. to stop phage maturation, and only those infected bacteria which contain at least one mature phage particle by the time streptomycin kills the bacteria are able to form plaques (Symonds, to be published).

Ultraviolet irradiation of infected bacteria. The method used for irradiating infected bacteria which had been withdrawn from an infected culture at different times during the latent period was similar to that described by Benzer (1952). To stop phage development at the required times, samples from the infected culture were diluted 100-fold into buffer chilled in an ice-bath. A 3 ml. volume from these samples was then irradiated with ultraviolet light on a watch-glass kept chilled by resting in a Petri dish full of crushed ice and water. The fraction of infected bacteria which survived the irradiation was determined from the number of irradiated bacteria that were still capable of originating plaques.

RESULTS

Mutant types arising in s *plaques*

When an *s* plaque is resuspended in buffer, and after an appropriate dilution, plated on agar plates, one always finds approximately equal numbers both of *s* plaques and typical *r* plaques. In some *s* plaques one also finds a few (< 1 %) particles that appear to be wild-type from their plaques, and also a few (< 1 %) other star variants that can be distinguished from the parental *s* because their plaques contain a smaller amount of sectored growth. These star variants were called 'derived star' or s_d mutants. The plaques of these s_d mutants contain about 90 % s_d phage and 10 % of typical *r* mutants. Two such s_d mutants were isolated whose plaques (Pl. 1) could be distinguished both from each other and from *s*. Fig. 1 shows schematically the way these various mutants originate from a single *s* plaque. In the succeeding sections we shall investigate the genetic structure of these mutants.

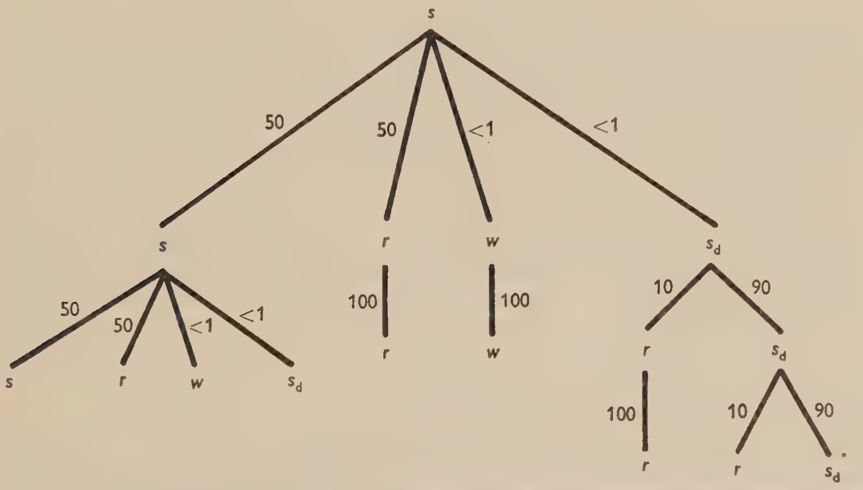

Fig. 1. Schematic representation of the different types of mutants that dissociate from a star plaque. The numbers refer to the percentage of each phage in the parent plaque.

Genetic structure of the s *mutant*

The genetic structure of the *s* mutant was first examined by performing a cross between *s* and wild-type. No recombinants were found in the progeny of this cross, which suggested that *s* was a one-step mutant of wild-type.

Further evidence supporting this idea came from crosses between *s* and reference mutants. At least 15% wild-type recombinants were found in the progeny of the crosses $s_1 \times r_7$, $s_1 \times r_{13}$, $s_1 \times m$ and 2·5% in the cross $s_1 \times r_1$. These results showed that the mutation responsible for the *s* phenotype behaved like a single locus that was located at a distance of 5 recombination units from r_1, and was thus unlinked to either r_7, r_{13} or *m*. We shall designate this star locus as s_1 (the subscript suffix 1 will be used to distinguish this from other star mutants). In Fig. 2 the location of s_1 on the T2 linkage map is shown with respect to the reference loci.

In the last four crosses described above only wild-type recombinants were observed. As will be shown later this is because the double recombinants $s_1 r_1$, $s_1 r_7$, and $s_1 r_{13}$ are phenotypically indistinguishable from *r* phage and thus could not be detected amongst the parental *r* phage in the progeny, while the double mutant *sm* is difficult to distinguish phenotypically from *m*.

Genetic structure of the ‘wild-type’ phage occurring in the s *plaques*

In order to see whether the wild-type plaques that were observed in the *s* plaques originated from phage particles that were genetically identical with wild-type, stocks isolated from these plaques were crossed with the standard wild-type stock. No recombinants were found in these crosses; it appears therefore that these plaques did originate from genuine wild-type phage.

Genetic structure of the s_d *mutants*

Further information that supported the identification of *s* as a one-step mutant of wild-type came from crosses performed with the two s_{d} mutants that had been isolated from *s* plaques. These two mutants could be distinguished by their plaque-type from *s*, *r*, and wild-type phage. When a cross was made between either of these s_{d} mutants and wild-type, equal numbers of two recombinants could be distinguished in the progeny. One of these recombinants was *s*, while the other was an *r* mutant. This finding indicated that the s_{d} were double-mutants of wild-type that contained, as well as a mutation at the s_1 locus, a mutation at some specific *r* locus. That is, the cross $s_{\mathrm{d}} \times$ wild-type could be represented:

	Parentals		Recombinants
Phenotype	$s_{\mathrm{d}} \times w$	$\rightarrow$	$s + r$
Genotype	$s_1 r \times s_1^+ r^+$		$s_1 r^+ + s_1^+ r$

A test was made of these genotypes by isolating the recombinant *r* mutant in the above cross, and then making the reverse cross $s \times r$. The progeny of this cross contained both wild-type and s_{d} phage particles with the same frequency of recombination as was found in the cross $s_{\mathrm{d}} \times w$. This is the result that would be expected from the proposed genotypes. The details of these crosses for both of the s_{d} mutants, which have been designated $s_1 r_{31}$ and $s_1 r_{32}$, are presented in Table 1 (in order to avoid confusion with the *r* loci already described by Hershey & Rotman (1949), we shall start numbering from 31 the *r* loci isolated from the star mutant).

Table 1. *Percentage of recombinants in crosses with two* s_d *mutants,* $\mathrm{s}_1\mathrm{r}_{31}$ *and* $\mathrm{s}_1\mathrm{r}_{32}$

Type of cross	Parentals	Percentage recombinants		Plaques scored
		s	r	
$s_d \times w$	$s_1r_{31} \times w$	2·6	2·9	1700
	$s_1r_{32} \times w$	14·5	16·0	550
		s_d	w	
$s \times r$	$s_1r^+ \times s_1^+r_{31}$	2·5	3·0	2500
	$s_1r^+ \times s_1^+r_{32}$	14·5	17·0	700

The location of the r_{31} and r_{32} mutations was found in two-factor crosses of the reference mutants with the recombinant r mutants $s_1^+r_{31}$ and $s_1^+r_{32}$. In this way r_{31} was shown to be linked to the locus r_1 with a frequency of recombination of 1 %, while r_{32} was unlinked to either r_1, r_7, r_{13} or m. The positions of r_{31} and r_{32} on the coliphage T2 linkage map are shown in Fig. 2.

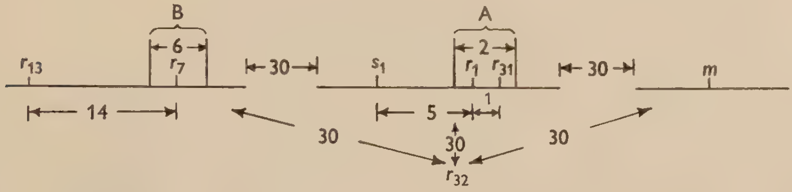

Fig. 2. Linkage map of coliphage T2 showing the locations of s_1 and of the r mutations occurring in the s_1r mutants. The numbers refer to the number of recombination units (i.e. the recombinant frequency in two-factor crosses) between the various loci. Approximately one-third of the r loci occurring in the s_1r mutants are in the A region, and two-thirds in the B (or rII) region. The indicated single-linkage group for coliphage T2 is based on the results of Baylor *et al.* (1957) and Streisinger (personal communication). Present evidence is insufficient to determine on which side of r_1 the s_1 locus is situated. Not drawn to scale.

Various checks can be made of these locations, and also of the genotypes that have been ascribed to s, s_d and the recombinant r mutants. First, it can be seen from Fig. 2 that the loci s_1 and r_{31} are approximately 6 recombination units apart, while the s_1 and r_{32} loci are unlinked. A verification of these map distances comes from the data of Table 1, where the frequency of recombination for the two-factor cross $s_1 \times r_{31}$ is given as 5·5 %, and that for the cross $s_1 \times r_{32}$ as 31·5 %. Secondly, it follows from Fig. 2 that the r_{31} and r_{32} loci should be unlinked. Experimentally this can be tested in two-factor crosses either between the r mutants r_{31} and r_{32}, or between the s_d mutants s_1r_{31} and s_1r_{32}. The first of these crosses resulted in 14 % wild-type recombinants as expected. In the second cross 16 % star recombinants were found as expected, and in addition 17 % r recombinants, which showed that the triple mutants $s_1r_{31}r_{32}$ had the r phenotype.

The results of these different crosses therefore all confirm the idea that the star mutant possesses a single mutation, s_1, while the s_d are double mutants with the genotypes s_1r.

Genetic structure of the r *mutants occurring in* s *and* s_d *plaques*

The genetic structure of the r mutants that were isolated from s plaques was next investigated. When any one of these r mutants was crossed against wild-type, s recombinants always appeared in the progeny. This finding suggested that these, like the s_d mutants, were double-mutants with the genotype s_1r. As the s_1^+r recombinant in the above cross could not be distinguished phenotypically from the s_1r parent, it was not possible to test the proposed genotype of these r mutants, as was done in the case of the s_d mutants, by performing the reverse cross $s_1r^+ \times s_1^+r$. However, the proposed genetic structure of these r mutants was verified, and the r loci of different mutants located, in 3-factor crosses with the reference mutants of the type $s_1r \times r_1$. From these crosses it was found that some of the r loci in the s_1r mutants were closely linked to r_1, while some were linked to r_7. Typical data for one such mutant, s_1r_{36}, in which the r_{36} locus is closely linked to r_7, is presented in Table 2.

Table 2. *Percentage of recombinants in crosses with an* r *mutant,* s_1r_{36}*, derived from an* s_1 *plaque*

Cross		Percentage recombinants		Plaques scored
		s	w	
$s_1r_{36}\times$	s_1	—	—	2000
	w	15·0	—	300
	r_1	11·5	1·5	1600
	r_7	0·2	0·2	7000
	r_{13}	6·0	5·0	1400

Table 3. *Percentage or recombinants in crosses with an* r *mutant,* $s_1r_{31}r_{63}$*, derived from a plaque of the* s_d *mutants* s_1r_{31}

Cross		Percentage recombinants			Plaques scored
		s	s_d	w	
$s_1r_{31}r_{63}\times$	s_1r_{31}	—	—	—	1400
	s_1	—	12·0	—	700
	w	1·0	10·0	—	1700
	r_7	0·1	0·5	0·5	1250
	r_{13}	0·2	3·3	2·7	1100

Except for the few wild-type phage particles, all the mutants that occur in the s plaques therefore appear to be double-mutants with the genotype s_1r. For most r mutations (including r_1, r_7, and r_{13}) these s_1r mutants have the r phenotype. However, there are some r mutations for which the s_1r mutants form plaques that still possess some star characteristics; these are the s_d mutants. Since the r mutants occurring in the s plaques still carry the parental s_1 mutation, one would expect that the r mutants arising, for example, in plaques of the s_d mutant s_1r_{31}, would still carry both the s_1 and the r_{31} loci. That is, they would be triple mutants with the genotype $s_1r_{31}r_a$. If this were so, in a cross between one of these mutants and wild-type both the s_d mutant s_1r_{31} and the star mutant s_1r^+ should be recognizable as recombinants. Table 3 presents the result of this and other crosses performed with one of these r mutants. These results are consistent with the idea that this is a triple mutant with the genotype $s_1 r_{31} r_{63}$ in which the r_{63} locus is closely linked to r_7.

The genotypes of the various mutants that dissociate from an s plaque are shown schematically in Fig. 3.

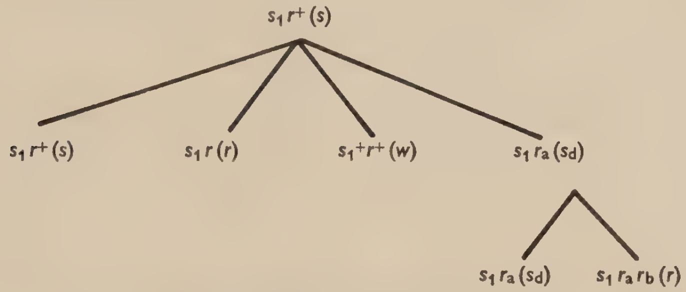

Fig. 3. Genotypes of the various mutants dissociating from the star plaques. The phenotypes of these mutants are shown in brackets.

Distribution of the r *mutations occurring in the* s *plaques*

In order to obtain some idea of the distribution of r mutations among the $s_1 r$ mutants found in s plaques, use was made of the discovery of Benzer (1956), that coliphages having a mutation in the rII region of the T2 chromosome (which includes the locus r_7), are not able to form plaques on plates seeded with the strain K12 (λ) of *Escherichia coli.*

First, a number of r mutants, all isolated from a single s plaque, were tested for their ability to form plaques on coli K12 (λ). Of 12 s plaques tested in this way, all yielded some r mutants that were able to form plaques on coli K12 (λ), and some that were unable to do so. This showed that at least two independent r mutations were present among the r mutants found in any one star plaque.

Next, 100 r mutants, each isolated from different s plaques (and therefore originating from independent mutations), were tested on coli K12 (λ). Approximately two-thirds of these were found to possess r mutations in the rII region. Ten of these mutants from the rII region were then crossed together in pairs. In all of these crosses s progeny were found, indicating that all 10 of these $s_1 r$ mutants possessed different r mutations. Ten of the mutants that were able to grow in coli K12 (λ) were then investigated further. It was found that their r mutations were all located within one recombination unit of the r_1 locus, and while 5 of the mutants were genetically distinct, 5 were either very closely linked or were identical.

In a parallel experiment performed on 100 spontaneous r mutants isolated from different wild-type plages, again approximately two-thirds of the mutations were found to be in the rII region (this is roughly the same distribution as Benzer (1956) found for spontaneous r mutations in the coliphage T4). Ten of the mutants that could grow on coli K12 (λ) were investigated in more detail, and found to be all genetically different, and to be all linked to r_1 with recombinant frequencies of between 0·5 and 4%.

On the basis of these results the distribution of the *r* mutations occurring in different *s* plaques is roughly similar to that found among the spontaneous *r* mutants. Since it has also been shown that the mutation rate from $s_1r^+ \rightarrow s_1r$ is approximately the same as the spontaneous rate $r^+ \rightarrow r$ (Visconti *et al.* 1953), it seems likely that the s_1r mutants found in the *s* plaques originate from spontaneous *r* mutations. Since an *s* plaque contains more than 10^8 phage particles, and since the mutation rate from $r^+ \rightarrow r$ is one in 10^3 duplications (Hershey, 1946), a number of independent *r* mutations will occur during the early growth of the plaque. If those s_1r mutants that are formed multiply at the same rate as *s*, then the *s* plaques would still contain only about 0·1% of these mutants. The high proportion of *r* mutants that do accumulate by the time the *s* plaques are fully grown must, therefore, be due to some efficient process of selection by which some at least of the s_1r mutants that have been formed can grow faster than the *s* phage under the physiological conditions holding during plaque formation. It is the purpose of the rest of this paper to investigate the mechanism which is responsible for this selection.

Growth of s *phage in broth cultures*

Since it was difficult to perform experiments during plaque formation that would give any information concerning the selective mechanism which enabled *r* phage to multiply faster than *s* phage in the star plaques, an attempt was made to determine growth conditions in broth cultures of bacteria in which there was a similar selective advantage for the multiplication of the *r* phage.

(*a*) *Growth of* s *and* r *phage in cultures of young bacteria.* First, the relative rates of multiplication of *s* and *r* phage were measured in a culture of young bacteria. A mixture of s_1r_{34} phage (which is a typical *r* mutant isolated from an *s* plaque, the r_{34} mutation being near the r_1 locus) and *s* phage was added to a culture of young bacteria. Antiserum was added 2 min. later, and after a further 6 min. (by which time the unadsorbed phage had been inactivated), a sample from this culture was diluted 500-fold into another culture of young bacteria. The subsequent rates of multiplication of the *s* and *r* phages in this culture was then followed by immediately assaying samples taken from the culture at various times after infection. The result of this experiment is presented in Fig. 4*a*. It can be seen that both the *s* and *r* phage multiplied in the young bacterial culture at roughly the same rate, and that when the culture lysed 80 min. after infection both types of phage had increased in titre 10^6-fold. During phage multiplication in young bacterial cultures the *r* phage therefore has no selective advantage over the *s* phage.

(*b*) *Growth of* s *and* r *phage in cultures of old bacteria.* The relative rates of multiplication of *s* and *r* phage in a culture of old bacteria was next measured in an experiment similar to the previous one. The result of this experiment is shown in Fig. 4*b* from which it can be seen that in the old bacterial culture the *s* phage multiplied much slower than the *r* phage . By 80 min. after infection, (which was the latest time any *s* phage particles could be detected in the culture), there was only an increase of 12-fold in the *s* titre compared to an increase of 2000-fold in the *r* titre. Because of this slow multiplication of *s* there is thus a strong selective advantage for *r* phage during phage growth in cultures of old bacteria.

These experiments show that it is possible to obtain growth conditions in broth cultures of bacteria in which *r* phage multiplies considerably faster than *s* phage. This selective advantage for the multiplication of *r* phage occurs during phage growth in cultures of old bacteria, but not in cultures of young bacteria. It occurs because the *s* phage multiply at a very slow rate in the old bacterial cultures.

Intracellular growth of s *and* r *phage in old bacteria*

One possible reason for the slow multiplication of *s* compared to *r* phage in old bacterial cultures was that there was a considerable delay in the time at which maturation of *s* phage particles began in old infected bacteria. Fig. 5 shows the result of an experiment to test this possibility, using the streptomycin technique. Although the $s_1 r_{34}$ infected bacteria formed their first plaques on the streptomycin plates about 2 min. earlier than the s_1 infected bacteria, there was no significant difference in the time at which phage development commenced in the greater proportion of the s_1 and $s_1 r_{34}$ infected bacteria. In a further experiment on a similar culture it was shown also that the number of intracellular s_1 and $s_1 r_{34}$ phage particles increased at approximately the same rate.

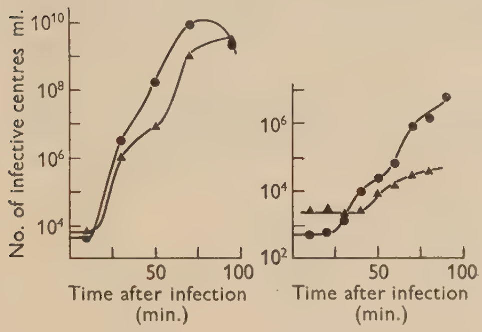

Fig. 4*a* **Fig. 4*b***

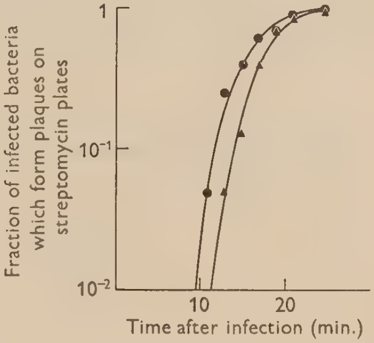

Fig. 5

Fig. 4. Growth of *s* (▲) and *r* (●) phage in the same bacterial cultures; (*a*) growth in young bacteria, (*b*) growth in old bacteria.

Fig. 5. Intracellular growth of *s* (▲) and *r* (●) phage in the same culture of old bacteria.

Intracellular *s* phage particles were thus produced at about the same time and rate in old bacteria as intracellular $s_1 r_{34}$ phage particles. The slow multiplication of *s* compared to that of *r* in a culture of old bacteria was therefore not due to any marked difference in the ability of the old bacterial cells to synthesize *s* and *r* phage particles.

Latent periods of s *and* r *phage in old bacteria*

Another possible reason why *s* phage multiplied slower than *r* phage in cultures of old bacteria was that in old bacteria *s* had a considerably longer average latent period than *r*. When a one-step growth experiment with *s* phage was performed in old bacteria some lysis occurred as early as 25 min. after infection, but the number of infective centres in the culture increased slowly over a period of 2 hr. In order to determine the average latent period of s it was thus necessary to estimate the fraction of the infected bacteria which had not lysed at any particular time after infection. Two methods, as described below, were used to obtain this information.

Antiserum inactivation of free phage. When assays are made, at different times after infection, from an infected culture to which strong antiserum has been added, there is a considerable increase in plaque count near the time when lysis is occurring (as many of the infected bacteria are then unstable and lyse during the dilution and warming to 45° that occurs before they can be plated), followed by a continual decrease in plaque count as more and more of the infected bacteria lyse and the particles they liberate are neutralized by the antiserum. Table 4 presents the result of an experiment in which this method was used to compare the latent periods of s_1 and s_1r_{34} phage in the same culture of old bacteria. Lysis of the s_1r_{34} infected bacteria is seen to have started by 24 min. after infection, and at 45 min. after infection the number of r infective centres had dropped to 10% of its original value; hence at least 90% of the s_1r_{34} infected bacteria must have lysed by this time. There is a slight increase in the titre of s_1 infective centres as early as 24 min. after infection, but the increase is most marked after 45 min. and still noticeable as late as 75 min. after infection. This result clearly indicates that the average latent period of s_1 in old bacteria is much longer than that of s_1r_{34}.

Table 4. *Number of* s_1 *and* $\mathrm{s}_1\mathrm{r}_{34}$ *infective centres present at different times after infection in a singly infected culture of old bacteria that lyses in the presence of strong antiserum*

Minutes after infection	Number of infective centres in a fixed dilution	
	s_1	s_1r_{34}
11	57	64
15	56	57
19	61	140
24	200	2000
30	400	400
45	3000	8
75	120	4

Irradiation experiment

It is possible to modify the previous experiment so that a more accurate estimate can be made of the number of s_1 infected bacteria that have not lysed after a certain period of time. It has been shown (Symonds, 1957) that at times later than 10 min. after infection the resistance to ultraviolet light of old bacteria infected with s_1 phage (i.e. the ability of these bacteria to produce at least one phage particle after irradiation), is much greater than the resistance of free T2 coliphage. Therefore if a sample is taken later than 10 min. after infection from an old infected culture of s_1 to which antiserum has been added, it can be irradiated with a dose of ultraviolet light that will inactivate any intra- or extracellular phage particles present in the sample, but have very little effect on the plaque-forming ability of the bacteria which have not lysed. The fraction of infected bacteria which still yield plaques after the irradiation then places a lower limit on the fraction of unlysed cells present in the culture at the time the sample was taken. Table 5 presents the result of an experiment of this kind in which the fraction of old bacteria infected with either s_1 or $s_1 r_{34}$ phage that survived an ultraviolet dose of 20 T2 lethal hits was compared at different times during the latent period. By 25 min. after infection only 3 % of the $s_1 r_{34}$ infected bacteria were still viable after the irradiation, compared to 90 % of the s_1 infected bacteria. Even as late as 50 min. after infection 30 % of the s_1 infected bacteria were still viable. Since these figures set a lower limit to the number of infected cells in the culture which have not lysed, they confirm the result that the latent period of s_1 in old bacteria is considerably longer than that of $s_1 r_{34}$.

Table 5. *Percentage of old bacteria, singly infected with* s_1 *or* $s_1 r_{34}$ *phage, that are still viable at different times after infection after being irradiated with an ultraviolet dose of 20 lethal T2 hits*

Minutes after infection	Percentage of infected cells that survive the irradiation	
	s_1	$s_1 r_{34}$
12	70	50
18	80	10
25	90	3
35	80	<1
50	30	<1

These experiments thus point to one reason why *s* phage multiplies so much slower than *r* phage in cultures of old bacteria. Since the latent period of *s* in old bacteria is longer than that of *r*, the intracellular *s* phage particles which are formed are unable to re-infect other bacteria in the culture as quickly as are the *r* phage particles; the *s* phage therefore carries out fewer cycles of growth and so multiplies more slowly in the culture.

Adsorption of freshly liberated s *and* r *phage*

An additional factor which might contribute to the slow multiplication of *s* phage in cultures of old bacteria was that the *s* phage particles which were eventually released when the cells lysed were unable to re-adsorb at the normal rate to other bacteria in the culture. This possibility was tested by infecting a culture of old bacteria with *s* phage and allowing phage growth to continue for 120 min. The culture was then centrifuged, and the rate of adsorption to old bacteria of the phage particles in the supernatant fluid was immediately measured. This supernatant fluid, and also a 1/10 dilution of it in buffer, were then stored at 4°, and the adsorption rates of both these phage suspensions were measured the next day. The results of two experiments of this type are presented in Table 6. In the 120 min. of phage growth the *s* titre increased about 100-fold. During this time the proportion of *r* phage in the cultures increased from 0·2 % in the original inoculum to be almost equal to that of the *s* phage. In the experiments the adsorption rates of both the freshly liberated *s* and *r* phage particles were therefore measured at the same time. The results show: (*a*) the ordinary stocks of *s* and *r* adsorbed at approximately the same rate; (*b*) the freshly liberated *s* phage particles adsorbed 15–25 times more slowly than freshly liberated *r* phage particles; (*c*) in the broth supernatants that were stored for 24 hr. there was little change in the adsorption rates of either *s* or *r*. However, in the dilute buffer suspensions the adsorption rate of *s* increased to about one-half that of *r*.

Table 6. *Percentage of* s *and* r *phage particles that are freshly liberated from old bacteria, which are able to re-adsorb to old bacteria in 5 min. at 37°. The control was a mixture of the standard stocks of* s_1 *and* s_1r_{34} *phage.*

	Type of phage		Percentage of adsorbing phage particles	
			s	*r*
First day	—	Control	10	7·5
	Supernatant of	Culture 1	3·5	50
		Culture 2	1·5	36
Second day	—	Control	10	6
	Supernatant of	Culture 1	3	20
		Culture 2	2·5	17
	1/10 buffer dilution of the supernatant of	Culture 1	18	20
		Culture 2	10	21

One plausible explanation for these findings would be that old bacteria infected with *s* phage do not liberate their intracellular phage particles abruptly at the end of their long latent period. Consequently pieces of bacterial membrane adhere to the phage particles as they are liberated and so hinder them from re-adsorbing to bacteria. However, on being left in dilute suspension for 24 hr. most of the bacterial debris dissociates from the phage particles, which then adsorb much more quickly.

These results, in conjunction with those of the previous sections, show that two factors contribute to the selective advantage which allows *r* phage to multiply much faster than *s* phage during growth in old cultures of *Escherichia coli*. First, in old bacteria the latent period of *s* is much longer than that of *r*, and secondly, the *s* phage particles that are eventually released from the old bacteria adsorb about 20 times more slowly to old bacteria than do freshly liberated *r* phage particles.

DISCUSSION

The star mutant discovered by Hershey and discussed in this paper is a single-step mutant of coliphage T2, having a mutation at a locus, s_1, that is close to the r_1 locus. This s_1 mutation has no effect on phage multiplication in cultures of young bacteria. However, it does have two probably related effects on phage growth in cultures of old bacteria. Under these conditions the star phage has a considerable extended latent period, and in addition the star phage particles which are eventually released from the old bacteria re-adsorb very slowly. These two effects cause the star phage to multiply at a very slow rate in cultures of old bacteria. Consequently any mutants that happen to be in the same old culture as the s phage, and can grow at the normal rate, have a marked selective advantage.

If it be assumed that these effects of the s_1 mutation also manifest themselves in the same way during plaque formation, then the characteristic appearance of the star plaques can be easily understood. While the bacteria on the plate are still young there is considerable multiplication of the star phage, and during this time a number of spontaneous mutations occur. As the bacteria on the plate become older, the s phage in the plaques multiplies more slowly, and any of the mutants that can multiply at the normal rate have a strong selective advantage. These mutants then grow out around the edge of the plaques forming clear sectors of growth which give the star plaques their characteristic appearance.

It seems probable that most other star mutants owe their distinctive plaques to some similar mechanism as this. That is, they have a mutation at one (or more) loci which exerts some inhibitory effect on phage multiplication under the conditions of plaque formation. Any spontaneous mutants that are formed in which this inhibition is released then have a strong selective advantage during the subsequent growth of the plaque. In star mutants in which the inhibitory effect is due to only one s locus there are two different kinds of mutation that can release the growth inhibition. The first kind is the back-mutation $s \rightarrow s^+$. This back-mutation automatically releases the growth inhibition and yields phage that no longer contains a mutation at the s locus. The other kind is a mutation at some further locus. This kind of mutation does not automatically release the inhibition, and always produces double mutants which still contain a mutation at the s locus. Whether or not these double mutants can multiply at the normal rate depends on both of the mutations involved. With the star mutant discussed in this paper, a further mutation at almost any of the numerous class of r loci yields s_1r phage that has the r phenotype and thus can grow normally. However, there are some r mutations which do not completely overcome the effect of the s_1 locus, and these produce 'derived star' plaques that still show some star characteristics. As the spontaneous mutations $s_1r^+ \rightarrow s_1r$ are more frequent than the back mutation $s^1 \rightarrow s_1^+$, most of the mutants found in these star plaques are r mutants which still contain the s_1 locus and have r mutations at different loci. However, it is possible with other star phage that mutants of the sr type are still growth inhibited. In these cases the phage selected for in the star plaques will either be due to back mutations, or to mutations at some other class of loci which are able to overcome the inhibitory effect of the s locus. An example of the first of these types is reported in the accompanying paper by McFall & Stent (1958). They have investigated a star mutant of coliphage T2 first isolated by

Litman, and find that the phage selected in these star plaques was all wild-type; in this case therefore the only frequent mutation that could overcome the inhibitory effect of the *s* locus was the back mutation $s \rightarrow s^+$. McFall & Stent have also investigated a star mutant of coliphage T2 discovered by Baylor (Baylor *et al.* 1957) which is of the second of the types mentioned above. In the plaques of this star phage the mutation which releases the inhibitory effect of the *s* locus is not an *r* mutation, but a mutation at a locus which lies very close to the *s* locus.

The mechanisms which cause growth inhibition in different star phages no doubt differ considerably. The one described here for s_1 is interesting in that it points clearly to two new factors which arise during the repeated cycles of growth that go to make up a lysate or a plaque, and which do not arise in one-step growth experiments. First, the physiological conditions of the host bacteria are continually changing; and secondly, the adsorbing phage particles do not come from a purified phage suspension but are always phage particles which have been freshly liberated from infected bacteria. It seems possible that these two factors might be important in understanding some processes of infection which occur in animal and plant virus systems.

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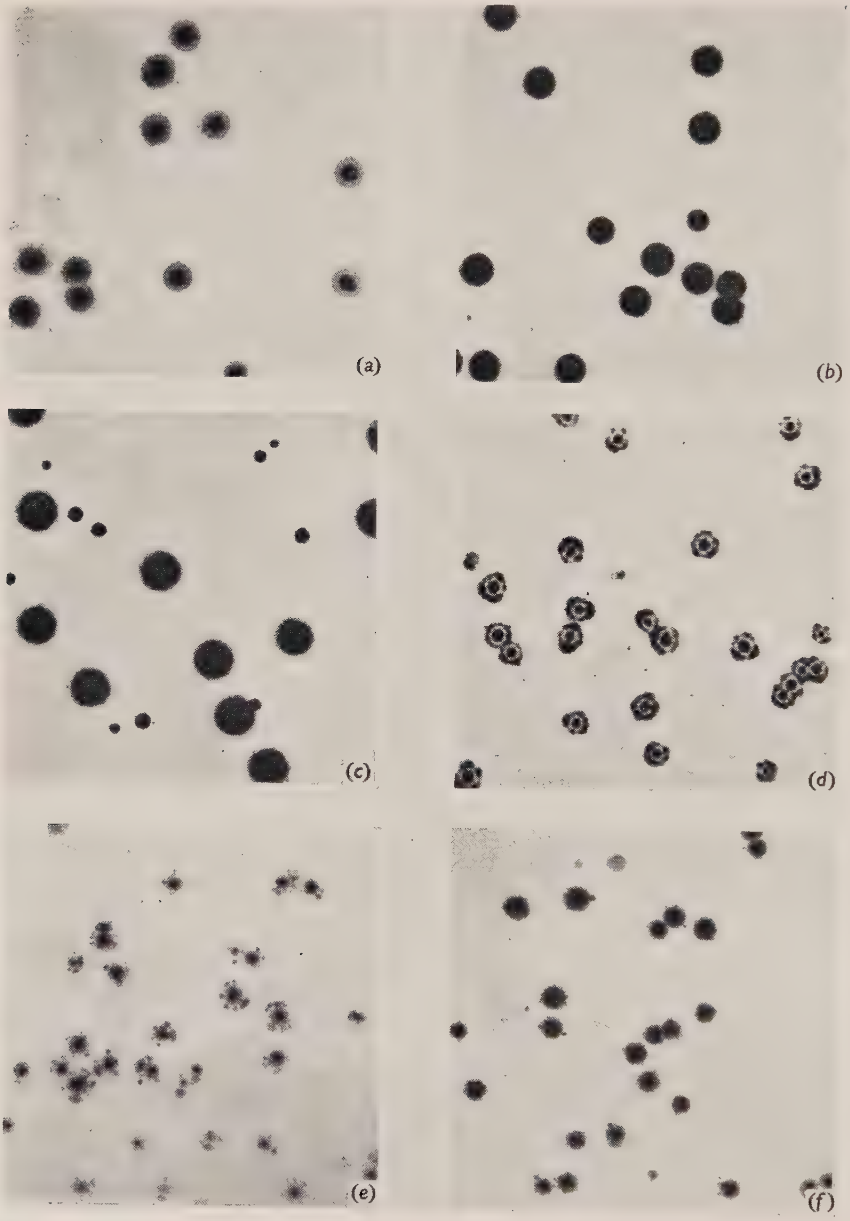

N. Symonds—A star mutant of phage T2. Plate 1

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EXPLANATION OF PLATE

Photographs of plaques of various coliphage T2 mutants. The different phenotypic types (with their corresponding genotypes) are: (*a*) wild-type (r^+), (*b*) r mutant (r_1), (*c*) mixture of minute (m) and an r mutant (r_1), (*d*) star mutant (s_1r^+), (*e*) derived-star mutant (s_1r_{31}), (*f*) derived-star mutant (s_1r_{32}).

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Three Star Mutants of Coliphage T2

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SUMMARY: Star mutants of coliphages produce irregularly-shaped plaques which harbour mixed phage clones consisting of two or more phage genotypes: one type which forms star plaques identical in morphology and genetic constitution to those of the primary star mutant and other types which form regular genetically-homogeneous plaques. The peculiar morphology and genetic heterogeneity of star plaques is due to the appearance and subsequent selection in every such plaque of secondary mutants with growth properties superior to those of the primary star mutant. In the present work, the genetic structure of three such primary star mutants of coliphage T2 and of the secondary, regular mutants which they sport has been examined. The 'bromouracil' star mutant s_8 owes its star character to a single mutation at a locus s_8, which is not closely linked to any known genetic marker of coliphage T2. The secondary, regular mutants found in every plaque of s_8 harbour *reverse mutations* to s_8^+ wild-type at the mutated star locus itself. The 'extended-host-range' star mutant h' owes its star character to the h' allele of the host range locus h. The secondary, regular mutants found in every plaque of h' harbour *suppressor* mutations to h' at a definite locus i_1 closely linked to h. These suppressor mutations abolish only the star effect of the h' allele without affecting the extended-host-range conferred by h' upon the coliphage T2. The 'P^{32}-decay' star mutant hr_s harbours a mutation r_s which extends over a considerable sector of the T2 genome situated between the distantly linked loci r_7 and r_1. The star phenotype of this mutant derives from an interaction between the host-range locus h and the r_s mutation so that some genetic states of the host range locus confer poor plaque development on phage particles in which they exist in conjunction with r_s. The secondary, regular mutants found in every plaque of hr_s harbour one of a set of non-allelic h^+ or ht mutations in the h locus, which abolish the plaque growth-inhibiting interaction of h with the r_s mutation and which also affect the host range. It is possible to replace variable sectors of the r_s mutation proximal to r_7 with their r^+ alleles by crosses of hr_s to wild-type. One may suppose that star mutants should be encountered with all those plaque-forming virus types which can sport with sufficiently high probability, i.e. soon enough during the development of each plaque, secondary mutants of growth characteristics superior to those of the parent type.

Bacteriophage particles inoculated on to nutrient agar plates seeded with sensitive bacteria produce circular areas of lysis called plaques. Although plaques formed by various types of bacteriophages differ greatly in size and general appearance, such plaques are usually perfectly circular, owing to the fact that bacteriophage growth and bacterial lysis proceed uniformly in all radial directions from the original focus of infection. Occasionally, however, plaques are observed which are very irregular, rather than circular, in outline. When such irregularly shaped plaques are picked and the phage clone so isolated replated, it is found that these plaques always contain two or more types of phage particles: one type which in turn gives rise to irregularly shaped plaques and other types which give rise to regular, circular, plaques. Phage variants which possess this peculiar plaque morphology and whose plaques are invariably mixtures of phage types have been observed with the following coliphages: T6 (Hershey, 1946); T2 (Visconti, Garen & Symonds, 1953; Baylor, Hurst, Allen & Bertani, 1957; Symonds, 1958); T4 (Doermann & Dissoway, 1949); T1 (Fuerst, 1954; personal communication); and with bacteriophages active on strains of *Salmonella* (Terada, Kondo & Ogawa, 1956). In agreement with Baylor *et al.* (1957) and Symonds (1958) we shall refer to such variants as 'star' mutants, although they have also been called 'unstable' or 'plaque-dissociation' mutants. Previous investigations have indicated that star mutants owe their irregular plaques and their 'dissociation' to a primary mutation which is prejudicial to reproduction of the mutant phage on the agar plate seeded with sensitive bacteria, so that plaques initiated by star mutants develop more poorly than those of the wild-type. At some stage of the development of every such star plaque, however, secondary mutant types make their appearance among the growing phage clone in which the growth inhibition of the primary mutation has been annulled and which mutants, therefore, possess a strong selective advantage over the parental star type during the remaining development of the plaque. Since these secondary mutants will usually appear at the periphery rather than at the centre of the plaque, their growth produces the eccentric areas of lysis which endow the star plaques with their characteristic irregular outline. In the present work, three such star mutants of coliphage T2 have been studied in order to elucidate the genetic structure of the primary mutations responsible for the plaque-growth inhibition and to investigate the nature of the secondary mutations which occur in the course of the development of each star plaque and which are responsible for annulling their growth inhibition.

METHODS

Bacterial strains. A number of different strains of *Escherichia coli* were used as hosts for coliphage T2 and its mutants: (These strains, which will be referred to in the following without the prefix *E. coli*, are: Strains S and B, H/2 (a mutant of B, H resistant to T2) isolated by A. D. Hershey; Strain B, H/2/*h* (a mutant of B, H resistant to T2h) isolated by M. B. Baylor; Strain B, B, isolated in our laboratory; Strain K12S(λ), referred to as 'K' in the following (an artificial lysogenic strain of K12S, isolated by E. Lederberg).

Bacteriophage strains. Beside the three star mutants of coliphage T2, described in detail in the text, the following mutants of T2(H) were employed: h^+r^+ (wild-type), hr^+, h^+r_1, h^+r_7, hr_{13}, and *m* (Hershey & Rotman, 1949), ht_6 (Baylor *et al.* 1957).

Media. Nutrient broth contains 8 g. dehydrated nutrient broth (Difco), 5 g. NaCl and 1 g. glucose/l. distilled water.

Phage assays. Phage assays were made on nutrient agar plates seeded with indicator bacteria, according to the methods described by Adams (1950).

Preparation of stocks of star mutants. It is not possible to prepare pure stocks of star mutant bacteriophages by the usual procedure of picking a mutant plaque and then inoculating a culture of sensitive bacteria with the contents of this plaque, since star plaques are always mixtures of star types and secondary mutants. Instead, pure stocks can be prepared by utilization of the single burst technique (Delbrück, 1945) in the following manner. A star plaque is picked and a bacterial suspension inoculated with the phage mixture at a low multiplicity of infection. After allowing 10 min. for phage adsorption, the suspension is diluted so that there is on the average one infected bacterium/ 1·5 ml. Samples (0·5 ml.) of this dilution are then placed into many individual culture tubes and incubated for 60 min. at 37° to permit phage growth and lysis of the infected bacteria. Half of the liquid volume of each tube is then plated on indicator bacteria and the plaque types present in each single burst tube noted. A single burst tube is then selected in which plating has revealed the presence of star mutants only, a growing culture of sensitive bacteria inoculated with the remaining half of the culture fluid of this tube, and the infected culture incubated until complete lysis has occurred.

Bacteriophage crosses. A culture of coli B, B in its logarithmic phase of growth in nutrient broth is infected with a tenfold multiplicity of each parent phage in the presence of 0·002 M-KCN. Phage adsorption is allowed to occur for 10 min. at 37° before any free phage remaining unadsorbed is neutralized with strong anti-T2 serum, and the culture is diluted at least 10^4-fold into broth to permit phage development. After 70 min. further incubation at 37°, the lysate is titrated on plates seeded with suitable indicator bacteria. The total burst sizes in these crosses are in the range of 100–200, except for crosses involving the marker m, when the burst sizes are sometimes as low as 50. Unless noted otherwise, the yields of the two parental types among the progeny are approximately equal. Recombinant frequencies greater than 1% are always based on the scoring of at least 100 plaques of each recombinant type. In crosses involving the marker r_7, wild-type r^+ recombinants are always scored by plating on coli S. The progeny of crosses involving the alleles h and h^+ are always pre-adsorbed to coli B, B before being plated on coli B, H/2 if the host range character is to be scored directly, in order to avoid complications due to phenotypic mixing (Novick & Szilard, 1951; Streisinger, 1956).

RESULTS

The 'bromouracil' star mutant s_8

A high proportion of plaque-type mutants is found among the progeny of T2 coliphages grown in a medium containing sulphanilamide and 5-bromouracil (Litman & Pardee, 1956), among which mutants star plaque formers can frequently be recognized. One such bromouracil-induced star mutant of phage r^+, isolated by R. M. Litman and referred to in the following as the 'bromouracil' star mutant, produces tiny plaques of irregular outline upon plating on coli S, as shown on Pl. 1, fig. *a*. When plaques of the bromouracil star mutant on coli S are picked and replated, they are found to contain approximately equal proportions of phages which again produce the original star plaques, and of phages which produce regular r^+ plaques, as shown in Pl. 1, fig. *b*. The plaques produced by secondary r^+ phages found in these star plaques are 'pure' in that they contain only regular r^+ types, whereas the secondary star plaques formed by star phages isolated from the primary star plaques once more contain equal proportions of both phage types. The bromouracil mutant is thus a typical star variant, in that a 'dissociation' into secondary mutants proceeds in all of its plaques. A pure stock of the bromouracil star mutant was prepared by the single burst technique described above. This stock, which constituted the descendants of a 10^8-fold multiplication of a star phage inoculum, contained a background of less than 0·1 % regular r^+ phages. The appearance of r^+ phages in its plaques cannot, therefore, reflect a very high intrinsic mutability of the star mutant. Rather, the bromouracil star mutant must owe its plaque morphology to a primary mutation responsible for poor plaque development and to the appearance and subsequent selection in each plaque of secondary mutants of superior growth properties.

Genetic structure of the star mutation. In order to determine the genetic structure of the primary star mutation, crosses were carried out with stocks of the bromouracil star mutant. The bromouracil mutant, first of all, was crossed to wild-type coliphage T2; among the progeny of this cross there appeared equal proportions of parental wild and star types and no discernible recombinants, suggesting that the primary star mutation of the bromouracil mutant concerns a single genetic locus. We shall designate this locus s_8 and refer to the bromouracil star mutant as s_8. Next, it was attempted to identify the situation of the mutated s_8 locus on the T2 linkage map by crossing the star mutant in five separate experiments to T2 phages bearing one of the markers r_1, r_7, r_{13}, m, or ht_6 (whose locations on the genetic map of T2 are indicated in Fig. 1) and scoring the percentage of wild-type, i.e. non-star r^+, non-star m^+, or non-star ht^+, recombinants appearing among the progeny. The following proportions of wild-type recombinants were produced in each of these crosses of s_8: against r_1:18 %; against r_7:25 %; against r_{13}:27 %; against m:32 %; against ht_6:17 %. It is thus seen that the percentage of wild-type recombinants produced in each of these crosses is so large that the star mutant locus s_8 cannot be closely linked to any of the five reference markers. (The fact that the percentage of wild-type recombinants produced in the cross to m exceeds the theoretical maximum of 25 % is probably due to the fact that the complementary double-mutant recombinant s_8m produces such tiny plaques on coli S that progeny of this genotype are overlooked in the estimate of the total burst size.) In a further experiment, s_8 was also crossed to a phage stock harbouring the r_s mutation described in a later section. Since wild-type non-star r^+ recombinants were produced also in the cross against r_s, it may be concluded that s_8 cannot be located in the r_s region between the distantly linked loci r_7 and r_1. It is, therefore, not possible to form an idea about the exact location of s_8 on the T2 genome, except to infer that s_8 is probably situated either to the left of ht_6 or to the right of m on the map shown in Fig. 1.

Genetic structure of the secondary mutants. Two possibilities suggest themselves concerning the nature of the regular r^+ phages found in the star plaques of s_8. Either these phages harbour reverse mutations from s_8 to s_8^+ or they still possess the s_8 allele but appear phenotypically as wild-types because of a secondary mutation at some other locus acting as a suppressor to the star phenotype expression of s_8. In order to decide between these two alternatives, five such secondary r^+ mutants were isolated from five different s_8 star plaques and crossed to T2 wild-type. If the secondary r^+ mutants still possessed the s_8 allele as well as a genetically distinct suppressor locus, then in a cross to wild-type it should be possible to reconstitute star mutants by recombining s_8 away from its presumed suppressor. No star plaques, however, were found among some 7000 progeny of each of these five crosses, indicating that the wild-type phages found in the star plaques of s_8 do not harbour suppressor mutations to s_8 but rather represent reverse mutants to s_8^+. The bromouracil star mutant is thus a much less complicated star type than the other two star mutants to be described in the following, in that original star mutation responsible for poor plaque development is eliminated during the growth of every plaque through a reversion at the mutated star locus.

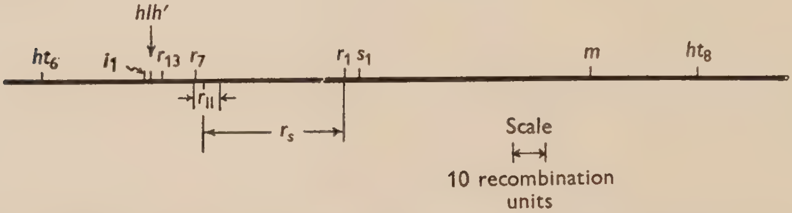


Fig. 1. The single linkage map of coliphage T2, showing the relative situations of the loci r_1, r_7, r_{13}, h, and m of Hershey & Rotman (1949), ht_6 and ht_8 of Baylor *et al.* (1957), s_1 of Symonds (1958), r_{II} of Benzer, as well as i_1 and the r_s mutation discussed in the present work. The indicated connection of Hershey & Rotman's linkage groups I, II and III is based on the studies of Baylor *et al.* (1957), Streisinger's three-factor cross (1955, personal communication), and the nature of the r_s mutation.

The 'extended-host-range' star mutant $h'r_1$

A mutant phage of extended host range was isolated by Baylor *et al.* (1957) by plating a high concentration of a stock of the host-range mutant hr_1 on coli strain B/2/h resistant to h. This mutant phage, designated as $h'r_1$, plates with equal efficiency on the three indicator coli strains S, B,H/2 and B,H/2/h; on the latter two of these indicator strains, phage $h'r_1$ forms regular r plaques, while on coli S the plaques of $h'r_1$ present the morphology of a typical star mutant shown in Pl. 1, fig. *c*. Baylor *et al.* found that the h' mutation is located within the h or host-range region, whose position on the T2 linkage map is indicated in Fig. 1 and whose fine structure has been studied by Streisinger & Franklin (1956). When star plaques of phage $h'r_1$ on coli S are picked and replated, they are found to contain approximately equal proportions of phages which again produce the original star plaques and of phages which produce regular r plaques, as shown in Pl. 1, fig. *d*. The plaques produced by the secondary r types found in star plaques are 'pure', in that they contain only regular r types, whereas the secondary star plaques formed by the star phages isolated from the original star plaques once more contain equal proportions of both phage types. On coli strain S, therefore, the 'extended-host-range' mutant phage behaves as a typical star mutant, in that it dissociates into secondary mutant types in all of its plaques. No dissociation, however, takes place in the r type plaques formed by phage $h'r_1$ on the other two indicator coli strains B,H/2 and B,H/2/h, for when such r plaques are picked and replated on coli S they are found to contain only phages which form star plaques. As in the case of the bromouracil star mutant, the dissociation of phage $h'r_1$ in its plaques on coli S cannot be due to an intrinsically high mutation rate, since pure stocks of phage $h'r_1$ can be grown on coli S in liquid medium without appearance of any appreciable proportion of r plaque formers.

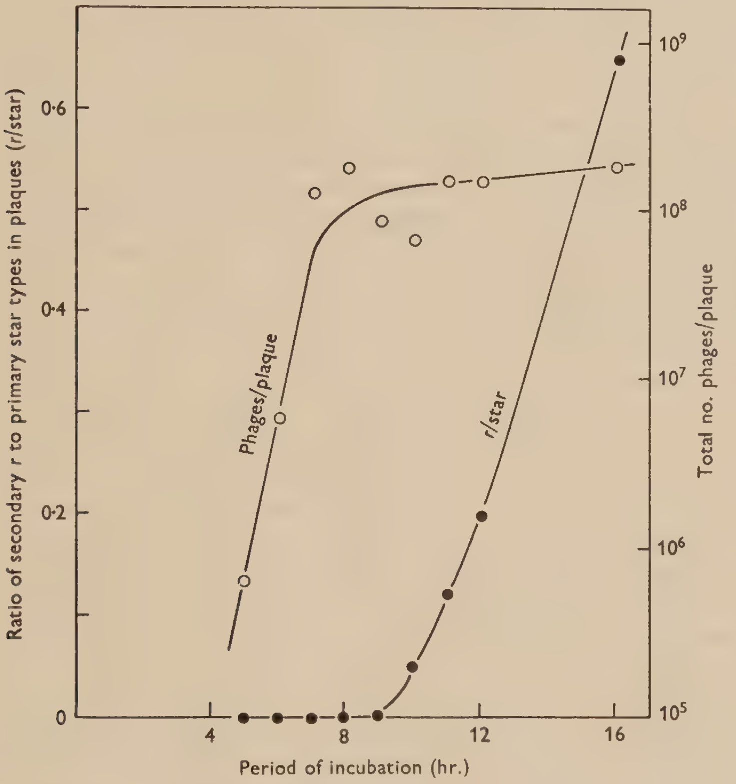


Fig. 2. Appearance of secondary r mutants in plaques of the extended-host-range star mutant phage $h'r_1$ on plates seeded with coli S.

Rather, the extended-host-range star mutant must also owe its star character to a primary mutation which causes poor plaque development on plates seeded with coli S, but not on plates seeded with coli B,H/2 or coli B,H/2/h, and to the appearance and subsequent selection in each plaque of secondary mutants of superior growth properties. In Fig. 2, the result of an experiment is presented in which individual plaques formed by phage $h'r_1$ on coli S have been picked and assayed for their contents of primary star and secondary r types at various stages of incubation, from the time at which the plaques first became visible until their final star morphology was manifest. It can be seen in Fig. 2 that by 8 hr., when the plaques had attained almost their final phage titre, practically no secondary r mutants were present, but that r mutants appeared only subsequent to this time, when multiplication of the primary star type had practically ceased.

Genetic structure of the star mutation. M. B. Baylor (personal communication) concluded on the basis of crosses of phage h' to other genetically marked strains of coliphage T2 that it is the h' allele itself which is responsible for the star character of the extended-host-range mutant. The following experiments confirm this inference. When phage $h'r_1$ was crossed to wild-type h^+r^+ and the progeny of this cross plated on coli S, about one-third of the phages were of the parental star and one-third of the parental r^+ plaque types. The remaining third of the progeny were comprised of equal proportions of two new, or recombinant, plaque types, one of these presenting the aspect of regular r and the other that of *minute* r^+ plaques, as shown on Pl. 1, fig. *e*. Several of the recombinant r plaques were picked and could be characterized as containing h^+r_1 phages, in that these recombinants failed to plate on coli B,H/2 and failed to yield wild-type recombinants in crosses to r_1. The *minute* r^+ recombinant plaques proved upon replating their contents on coli B,H/2/h to contain only h' genotypes and upon replating on coli S to be of a star character, in that they contained a mixture of equal proportions of *minute* r^+ and regular r^+ plaque formers, Pl. 1, fig. *f*. The result of this cross shows that the mutation responsible for the star character of phage $h'r_1$ behaves as a simple genetic locus in crosses and that it confers the star character not only in conjunction with the r_1 gene but also in r^+ phages. The fact that all the *minute* r^+ recombinants examined were of h' genotype suggests that the star mutation in phage $h'r_1$ occurred at a locus near to or identical with that of h'.

A pure stock of one of the minute $h'r^+$ recombinants produced in the above cross was prepared by means of the single burst technique and a number of further crosses carried out with this recombinant to ascertain the location of the star mutation. First, the $h'r^+$ recombinant was crossed to h^+r_1, a cross reciprocal to that which had given rise to this recombinant in the first place. The progeny of this reciprocal cross were plated on coli S and were found to consist of 70 % parental types and of 30 % r_1-star and wild-type h^+r^+ recombinants in equal proportions. Picking a number of recombinant r_1-star plaques and replating their contents on coli B,H/2/h showed that all contained only h' phages, i.e. that a genotype corresponding to the original extended-host-range star mutant $h'r_1$ had been reconstituted by the reciprocal cross and that the star mutation must reside at a locus close to or identical with h'. This inference was confirmed by a set of four experiments in which the $h'r^+$ recombinant was crossed to T2 phages bearing one of the markers m, r_1, r_7, or r_{13} and the percentage of wild-type, i.e., non-star r^+ or non-star m^+, appearing among the progeny scored. The following proportions of wild-type recombinants were produced in each of these crosses of $h'r^+$: against r_1:20 %; against r_7:9·7 %; against r_{13}:1·1 %; against m:33 %. It is thus evident that a sufficiently high percentage of wild-type recombinants are produced in crosses to m and r_1 to indicate that the star mutation occurred at a locus only distantly linked to these two markers. The lower percentages of wild-type recombinants issuing from the crosses to r_7 and r_{13} show that the star mutation must have approximately the same genetic location as h', which is situated within 1·1 % recombination from r_{13} (Baylor *et al.* 1957). The recombinants reciprocal to wild-type in the crosses of $h'r^+$ to h^+r_7 and h^+r_{13} exhibited the morphology of r-star plaques, indicative of the fact that the extended-host-range star mutation confers the star character also on phages harbouring the r_7 and r_{13} mutations.

Genetic structure of the secondary mutants. The same two possibilities concerning the origin of the regular, non-star phages found in every star plaque which have already been discussed in connexion with the bromouracil star mutant may now also be considered for the extended-host-range star mutant. In order to distinguish whether secondary mutants of superior plaque development arise by reverse mutations of the star locus itself or by suppressor mutations at some other locus, four secondary, non-star mutants were isolated from two star plaques formed by the original extended-host-range star mutant $h'r_1$ and from two star plaques formed by the recombinant $h'r^+$. Plating on B, H/2/h revealed that all four of these secondary mutants had retained their h' genotype, i.e. that the mutations which had abolished the star character had not altered the host-range phenotype of the h' locus. Each of these four secondary non-star mutants was then crossed separately to h^+r^+ wild-type and the progeny of this cross scored on plates seeded with coli S. The result of these crosses was that about 1 % of the progeny of each cross were found to have regained the star character. This reconstitution of star phages in crosses of wild-type to mutants which have lost their star phenotype can only mean that the secondary non-star mutants isolated from star plaques harbour both the original star mutation and a suppressor mutation i_1, located within a distance of about 2 recombination units from the h' locus. The reconstituted star types of these crosses thus represent recombinants which possess the i_1^+ allele of the wild-type parent and the h' allele of the secondary mutant. In order to examine whether the suppressor mutation i_1 had, in fact, occurred at the same locus in all four of these secondary non-star mutants, each mutant was crossed against every other mutant, since star types should be found among the progeny of such crosses if the four independent suppressor mutations were non-allelic. No star recombinants were found in any of these crosses, however, from which it may be inferred that allelic suppressor mutations i_1 had occurred in all four secondary non-star mutants. In order to place more exactly the location of i_1 relative to that of h', one of the secondary non-star mutants of genotype $i_1h'r^+$ was crossed to h^+r_{13}. Among the progeny of this cross there appeared about 3·0 % r^+ star recombinants and many fewer, if any, r star recombinants. This indicates that i_1 is not located on the same side of the host range locus as r_{13}, i.e. that the order i_1–h'–r_{13} must be ascribed to the three loci involved.

Thus, the extended-host-range mutant of Baylor *et al.* appears to represent a phage in which the mutation to h' at the h locus has modified the phenotype in such a way that although the particle can be adsorbed to coli B, H/2/h, its plaque development on coli S has become impaired. A secondary mutation at a definite locus i_1, linked to h' and situated on the other side of the host range locus from r_{13}, occurs during the growth of every plaque. This secondary mutation suppresses the deleterious effect of h' on plaque development and restores normal plaque growth on coli S to phage particles of the $i_1 h'$ genotype without affecting the beneficial effect of h' on host range.

The 'P^{32}*-decay*' *star mutant* hr_s

Among the survivors of radioactive decay of a stock of phage hr^+ heavily labelled with P^{32} a star mutant was observed whose characteristic irregular plaque morphology of sectoring and large centre, shown in Pl. 1, fig. *g*, render it easily distinguishable from normal r^+ plaques of the wild-type, as well as from the other two star mutants described above. The 'P^{32}-decay' star mutant develops its most characteristic star plaques on plates seeded with 24 hr. cultures of coli B,B, forms less pronounced, though still recognizable star plaques on coli S, and produces large, non-star *r*-type plaques on coli B,H/2 with an efficiency of plating near unity. As with other star mutants, picking and replating of star plaques of the P^{32}-decay mutant shows them to be a mixture of two phage types in approximately equal proportions. One of these types again gives rise to star plaques and the other produces the regular *r* plaques shown in Pl. 1, fig. *h*. The secondary *r* plaques are 'pure', in that they contain only regular *r* types, whereas the secondary star plaques isolated from the primary star plaques once more contain equal proportions of both phage types. Like that of the other two star mutants described above, the dissociation into secondary mutant types in all plaques of the P^{32}-decay star mutant cannot be due to an intrinsic high mutability, since high-titre stocks of the P^{32}-decay star mutants containing a negligible background of secondary *r* types can be grown on coli B,B in liquid cultures from small star phage inocula by means of the single burst isolation technique. Hence the P^{32}-decay star mutant must also owe its star character to a primary mutation which causes poor plaque development on plates seeded with coli B,B and to the appearance and subsequent selection in each plaque of secondary mutants of superior growth properties. As the experiments to be described in the following show, however, the genetic structure of this primary mutation and the mechanism of the release of its plaque-inhibiting effect is very much more complicated in this mutant than in the case of other star mutants previously described.

Genetic structure of the star mutation. Table 1 presents the result of a cross of the P^{32}-decay star mutant to h^+r^+ wild-type. It may be seen that only 16% of the progeny could form plaques on coli B,H/2, i.e. were of *h* genotype, in spite of an equal input of *h* and h^+ parents. This discrepancy in yield between the alleles introduced by the two parents appears to be a consequence of the poor growth properties of the P^{32}-decay star mutant (whose burst size rarely rises above 20 in single infection on coli, B,B) and complicates any exact quantitative inferences. The distribution of plaque types exhibited on coli B,B by the progeny of this cross reflects the same disparity in yield of parental alleles, since 78% of the progeny were of r^+ genotype, while 12% register as star and 10% as the *r*-type phages shown in Pl. 1, fig. *i*. Nevertheless, certain conclusions can be drawn from these results. The appearance of *r* recombinants among the progeny of this cross indicates, first of all, that the P^{32}-decay star mutant harbours an r mutation; we shall designate this mutation r_s and refer to the P^{32}-decay star mutant as phage hr_s. The phenotypic expression of r_s as r plaque type, furthermore, appears to be inhibited by some other genetic locus present in the star mutant and absent in wild-type, from which locus r_s has been separated in the r recombinants produced in this cross. The fact that the frequency of r recombinants is as high as 10%, compared to a yield of 12% of the parental star types, indicates that r_s and its inhibitor cannot be closely linked. Fifty plaques of star and r types formed by the progeny of this cross on coli B, B were picked and replated on coli B, H/2 in order to score also their host-range character. The results of this test are likewise presented in Table 1, where it can be seen that, as with the parent star type, all the progeny star types were h, while all of the r recombinants were h^+. This suggests that the locus which inhibits expression of the r phenotype is closely linked to the h locus. As further experiments described below show, the star character of phage hr_s arises from the interaction of this h-linked inhibitor locus with the r_s mutation. In order to determine whether the primary event which created the P^{32}-decay star mutant out of phage hr^+ by producing the r_s mutation also entailed a second genetic disturbance producing an inhibitor for r_s near h, or whether this inhibitor already exists in phage hr^+, the hr_s star mutant was crossed to hr^+. Among the progeny of this cross plated on coli B, B there appeared 75% r^+ and 25% star types, but no r recombinants, demonstrating that the locus inhibitory to the expression of r_s as r is already present in the host-range mutant h. (The unequal yields of parental types issuing from this cross reflect once more the inferior growth properties of the P^{32}-decay star mutant.)

Table 1. *Progeny of the cross* P^{32}-*decay star mutant* × *wild-type* ($T2hr_s \times T2h^+r^+$)

	Host range			
	Plaque formers		Genotypes	
Plaque morphology	On coli B, H/2 (%)	On coli B, B* (%)	h (%)	h^+ (%)
r^+	4·0	78	4·0	74
Star	12	12 (50)	12	<0·4
r		10 (0)	<0·2	10
Total	16	100	16	84

* The figures in parentheses indicate the number of plaques out of fifty picked at random whose phage clone was found to plate on coli B, H/2.

A number of experiments were carried out in order to locate the r_s mutation on the T2 linkage map. An indication of the probable position of the r_s mutation is already given by the observation that although the original wild-type T2 phage forms plaques with an efficiency of 0·1 on the bacterial indicator coli strain K, less than 10^{-6} of hr_s star mutant individuals can plate on this strain. It seems likely, therefore, that the mutation r_s is situated in the r_{II} region of the phage genome where that class of mutations occur which confer upon mutant phages the r phenotype when plated on coli S and prevent their multiplication on coli strain K (Benzer, 1955, 1957). The hr_s star mutant was now crossed in three separate experiments to h^+ phages bearing one of the markers r_1, r_7 or m, and the percentage of wild-type (i.e. non-star r^+ or non-star m^+) recombinants among the progeny scored. The following proportions of wild-type recombinants were produced in each of these crosses of hr_s: against r_1:0·1 %; against r_7:1·0 %; against m:15 %. The result that in the cross to r_7 very few and in the cross to m very many wild-type recombinants appeared is in agreement with our previous supposition that r_s is in the r_{II} region, and hence closely linked to r_7 and only distantly linked to m (cf. Fig. 1). Contrary to expectation, however, extremely few wild-type recombinants appeared also in the cross to r_1, a locus very distant from r_7 and the r_{II} region. The primary mutation r_s thus appears to be closely linked to a pair of markers which exhibit only very distant linkage to one another.

Table 2. *Genetic properties of T*2hr$_s$ *and various* r*-type recombinants produced by the cross T*2hr$_s$ × *T*2h$^+$r$^+$

Phage strain	Efficiency of plating on coli strain K	Percentage r^+ recombinants among progeny of cross against	
		h^+r_7	h^+r_1
hr_s	$<10^{-6}$	1·0	0·1
r_{s1}	0·1	17	0·1
r_{s2}	—	18	0·1
r_{s3}	—	11	0·4
r_{s4}	—	17	0·5
r_{s5}	—	20	0·8
r_{s6}	—	6·0	0·5
r_{s7}	0·1	16	0·4
r_{s8}	$<10^{-4}$	3·0	0·1
r_{s9}	0·1	8·0	0·2
r_{s10}	$<10^{-4}$	1·0	0·1
r_{s11}	0·1	16	0·2
r_{s12}	0·1	15	0·1
r_{s13}	0·1	18	0·2

Two alternative explanations offer themselves for this unexpected linkage of r_s to the two distantly linked markers r_1 and r_7. It is, on one hand, possible that r_s represents not one but two 'point' mutations in the primary P^{32}-decay star mutant, one having occurred in the r_{II} region and the other in the immediate vicinity of r_1. On the other hand, r_s could represent a long region of extensive genetic modification, extending all the way from the r_{II} region to r_1 similar to, but much longer than, the r 'deletions' discovered by Benzer (1957) in coliphage T4. It was possible to decide between these two alternatives through genetic analysis of the r recombinants which appeared in the cross $hr_s \times h^+r^+$. Several of these r recombinants were isolated and a number assigned to each. Some of these recombinants were tested for their efficiency of plating (e.o.p.) on the indicator coli strain K, and the result of this test is shown in Table 2. It can be seen that six out of eight recombinants examined exhibit as high an e.o.p. on coli strain K as the T2 wild-type phage, while the remaining two recombinants exhibit as low an e.o.p. on coli strain K as the parent star hr_s phage. The first six recombinants, though of r genotype, must, therefore, harbour no longer that part of r_s which is situated in the r_{II} region. This result is, of course, in agreement with either alternative and confirms only that r_s is complex in that the primary star mutation affected loci both within and without the r_{II} region. The various r recombinants were now crossed to h^+r_7 and the percentage of r^+ wild-type phages among the progeny scored. The result of these crosses is also presented in Table 2, where it may be seen that different r recombinants show a variable linkage to r_7, since some produce very few and some produce many r^+ wild-types against r_7. In agreement with our previous inference, it is seen that those two r recombinants which fail to plate on coli strain K are closely linked to r_7, while those six recombinants which do plate on K are more distantly linked to r_7. The fact that different r recombinants present a spectrum of linkages to r_7 indicates that, like Benzer's T4 'deletions', the r_s mutation has, in fact, altered a long portion of the T2 genome and that different r recombinants harbour different segments of this r_s mutant region, depending on the points at which there occurred those cross-overs between wild-type and hr_s genome which created the particular recombinant. The same set of r recombinants was also crossed against phage h^+r_1 and the percentage of r^+ progeny issuing from each cross scored. The result of this second series of crosses is likewise presented in Table 2, where it may be seen that only a very low percentage of r^+ progeny appear from the cross of any r recombinant against r_1, indicating that all of these r recombinants still harbour that part of the r_s region which is proximal to r_1. Finally, a number of r recombinants exhibiting different linkages to r_7 were intercrossed against each other and the appearance of r^+ wild-types sought. In none of such intercrosses between r recombinants, however, could any r^+ progeny be detected, indicating that all of these r recombinants still harbour common or allelic regions of the r_s mutation. The genetic structure envisioned on the basis of these results for r_s and for the various r recombinants produced in the cross of the hr_s star against h^+r^+ wild-type is presented in Fig. 3.

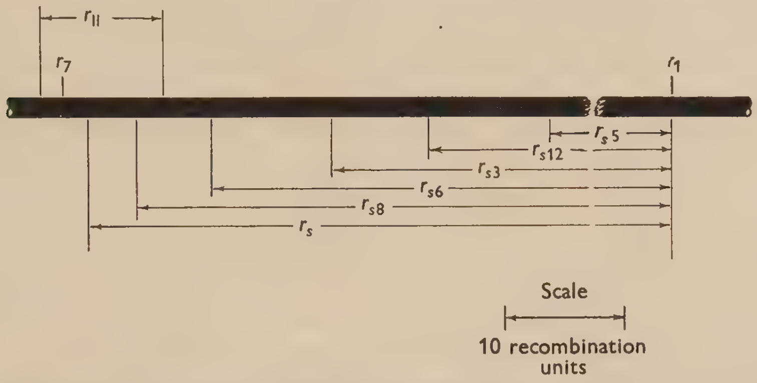


Fig. 3. The region of the linkage map of coliphage T2 between the markers r_7 and r_1, showing the portions of the r_s mutation present in various r recombinants produced in the cross $hr_s \times h^+r^+$.

A rather surprising aspect of the experiments presented in Table 2 is that all of the thirteen r recombinants isolated from the cross $hr_s \times h^+r^+$ exhibit close linkage to r_1, i.e. that no r recombinant was found in which the r^+ alleles of the wild-type parent have replaced the section of r_s mutation proximal to r_1 and which retain only the r_{II} section of r_s proximal to r_7. It is conceivable that no such recombinants are found because phages in whose genome only the r_{II} part is mutated might not exhibit the r but rather the star phenotype, and thus, though present among the progeny of the cross $hr_s \times h^+r^+$, be mistaken for parental star types. In order to examine this possibility, twenty star plaques found among the progeny of this cross were picked, the star phage contained in each plaque isolated by the single burst technique, stocks of these star phages crossed to hr_1, and r^+ wild-types among the progeny scored. The result of this experiment was that only between 0·1 and 0·5 % r^+ recombinants appeared among the progeny of any of these crosses, indicating that all the star progeny of the cross star × wild tested still harbour a mutation proximal to r_1. It would follow, therefore, that among the progeny of the cross star × wild few, if any, r recombinants are found in which only the part of r_s close to r_7 is in mutant form, not because such genotypes are star phenotypes, but because such recombinants are either not formed or not viable.

It was inferred above that the star character of phage hr_s is due to the inhibitory effect of a locus closely linked to h on the expression of r_s as an r phenotype, since all the r recombinant progeny of the cross $hr_s \times h^+r^+$ were of h^+ genotype and since only parental types but no r recombinants were produced in the cross $hr_s \times hr^+$. Since the gentic analysis of the r recombinants of the cross $hr_s \times h^+r^+$ has shown that many of these recombinants contain only a *part* of the r_s mutation, it may be concluded from the absence of r types among the progeny of the cross $hr_s \times hr^+$ that the h-linked locus can exert its inhibitory effect on the expression of the r phenotype not only in phages which harbour the entire r_s mutation but also in recombinants in which only a part of r_s is present. Hence among the star-type progeny of the cross $hr_s \times hr^+$ there should exist some recombinants in which a fraction of the r_s mutation has been replaced by the wild alleles of the r^+ parent. In order to test this prediction, a number of star plaques formed by the progeny of the cross $hr_s \times hr^+$ were picked and pure stocks of the star types within these plaques prepared by the single burst technique. The efficiency of plating on K of these star progeny was determined and each isolate crossed to h^+r_7 in order to measure the frequency of r^+ progeny produced. The results of this experiment are presented in Table 3, where it may be seen that two out of the six stars isolated have an e.o.p. on coli strain K of 0·1, i.e. the r_{II} part of r_s is no longer present, and shows less linkage to r_7 than the parental hr_s star mutant. (We can offer no ready explanation for the curious result that two of these progeny stars, i.e. nos. 2 and 4, show somewhat closer linkage to r_7 than the parent star.) The fact that these two recombinants still exhibit the star phenotype while harbouring only part of the r_s mutation then confirms our previous notions concerning the inhibitory power of the h-linked genetic locus. The inhibitory effect of this locus on plaque development of phages bearing only part of the r_s mutation was also demonstrated by crossing hr^+ phages to the recombinants

r_{s1}, r_{s2}, and r_{s9} described in Table 2, recombinants which were shown previously to lack portions of the r_s mutation proximal to r_7. In all three of these crosses there appeared from 8·0 to 13% star types among the progeny, demonstrating that the star character can indeed be reconstituted by re-introducing the locus closely linked to h into phages harbouring only parts of r_s.

Table 3. *Genetic properties of various star-type progeny issuing from the cross* $T2hr_s \times T2hr^+$

Progeny star	Efficiency of plating on coli strain K	Percentage r^+ recombinants among progeny of cross against h^+r_7
1	0·1	11
2	$<10^{-6}$	0·1
3	$<10^{-6}$	2·0
4	$<10^{-6}$	0·3
5	0·1	8·0
6	$<10^{-6}$	1·0

Genetic structure of the secondary mutants. In order to examine the genetic structure of the secondary regular r mutants present in every plaque of the P^{32}-decay star mutant phage hr_s, a number of such star plaques were picked and the secondary r mutants contained therein isolated and grown into pure stocks. These secondary r mutants possess a plaque morphology somewhat different from that of the r recombinants produced from the cross $hr_s \times h^+r^+$, as may be seen in Pl. 1. Each of the r mutants isolated was plated on the indicator coli strain K and three of the mutants crossed separately to h^+r_1 and h^+r_7 and the percentage of wild-types among the progeny scored. The results of these tests are shown in Table 4, where it may be seen that all of the 8 r mutants isolated exhibit a very low e.o.p. on coli strain K, i.e. in all of them the r_{II} portion of r_s is still present, and that the 3 mutants tested in crosses to r_1 and r_7 produced wild-type r^+ recombinants to the same degree as the primary hr_s star mutant phage. It may be concluded, therefore, that unlike the r recombinants, the secondary, regular r mutants still harbour all of the primary r_s mutation. The same set of secondary r mutants was now plated on the indicator coli strain B, H/2 in order to examine their host-range phenotype. The result of this test is also presented in Table 4, where it may be seen that the host range of all of these secondary mutants is very different from that of the primary star phage, in that these mutants either do not plate at all on coli B, H/2, or still do so only to form very turbid instead of clear plaques. It appears, therefore, that the secondary mutations which create the regular r phenotype out of the primary star phage affect the h character. This observation is not only in harmony with our previous conclusion, based on the results of crosses of hr_s against h^+r^+ and hr^+, that the star character of the P^{32}-decay mutant derives from the inhibitory action on the expression of r_s as an r phenotype of a locus closely linked to h, but indicates that this locus is, in fact, the h allele itself. For it appears from the results presented in Table 4 that the secondary r mutants found in every star plaque arise from mutations within the h region, mutations which alter the character of that region in such a way that it no longer confers poor plaque-growth characteristics on phages which harbour the entire r_s region and which mutations cause at the same time a reduction of host range. We shall refer to the mutated host-range loci of secondary mutants of hr_s either as h^+ (following Streisinger & Franklin, 1956) if the e.o.p. on coli B, H/2 of the mutant is very low or as *ht* (following Baylor *et al.* 1957) if the mutant forms turbid plaques on coli B, H/2. A number has been assigned to each secondary mutant, so that different secondary mutants of regular r phenotype isolated from star plaques of hr_s bear such descriptions as $h^+_{s1}r_s$ or $ht_{s4}r_s$ (cf. Table 4).

Table 4. *Genetic properties of various secondary mutants isolated from star plaques of* hr$_s$

Phage strain	Efficiency of plating on coli strain K	Percentage r^+ recombinants among progeny of cross against		Efficiency of plating on strain B, H/2
		h^+r_7	h^+r_1	
hr^+	0·1	—	—	1·0
hr_s	$<10^{-6}$	1·0	0·2	1·0
$h^+_{s1}r_s$	$<10^{-6}$	1·0	0·4	$<10^{-7}$
$h^+_{s2}r_s$	$<10^{-6}$	—	0·1	$<10^{-7}$
$h^+_{s3}r_s$	$<10^{-6}$	1·3	—	$<10^{-7}$
$ht_{s4}r_s$	$<10^{-6}$	2·0	0·2	1·0 tu*
$ht_{s5}r_s$	$<10^{-6}$	—	—	1·0 tu
$ht_{s6}r_s$	$<10^{-6}$	—	—	1·0 tu
$h^+_{s7}r_s$	$<10^{-6}$	—	—	$<10^{-7}$
$h^+_{s8}r_s$	$<10^{-6}$	—	—	$<10^{-7}$

* tu = turbid plaques.

The reverse mutations of h to inferior host range observed here are similar to those found by Streisinger & Franklin (1956) in the study of the fine structure of the h locus of phage T2, in that the sites affected by mutation are, in general, non-allelic though closely linked. This can be demonstrated by crossing the pair of the secondary r mutants $h^+_{s1}r_s$ and $h^+_{s2}r_s$ described in Table 4. Among the progeny of this cross there appeared 0·5% recombinants to whom the h host range had been restored, and which once more exhibit the star phenotype when plated on coli B, B. This concomitant reconstitution of h host range and star character by crosses of independent h^+ reverse mutants thus provides further evidence that it is the conjunction of the h allele with the r_s mutation which confers the star character on the P^{32}-decay mutant. Star types can be reconstituted not only by crosses of independent secondary mutants but also by further mutation of secondary mutants, as may be demonstrated by picking the turbid plaques formed on coli B, H/2 by such secondary r mutants as $ht_{s4}r_s$ and replating the phage clone on coli B, B and coli B, H/2. For it is found that these turbid plaques contain a high proportion of phages to whom not only the full h host range but also the star character has been restored. These reconstituted star types represent the descendants of tertiary mutants in which there occurred a reverse-reverse mutation from *ht* to h and which mutants were subsequently selected in the course of further plaque development on coli B,H/2 because of their superior host range. Star types, finally, can also be reconstituted by reintroducing by recombination an intact h allele into secondary mutants sported by hr_s. For instance, when the secondary mutants $h_{s1}^+ r_s$ and $ht_{s4} r_s$ described in Table 4 were each crossed against hr^+, there appeared respectively 9 and 10% star types among the progeny. Such reconstituted star types evidently represent recombinants which harbour the h allele of the hr^+ parent and all, or part of the r_s deletion of the hr_{s1}^+ or $ht_{s4} r_s$ parent. By utilization of the modified single burst technique of Luria (1951) an estimate has been made of the rate at which phage hr_s sports those mutations in its h locus which produce secondary r phenotypes. In this experiment there appeared 45 clones of secondary r mutants among 42, 700 hr_s progeny phages. Since the mutation rate is the ratio of the number of mutant clones to twice the number of phage particles among which these clones have arisen (Luria, 1951), a mutation rate of 5×10^{-4} mutations/phage particle/duplication may be inferred from these results, in reasonable agreement with previous estimates of the mutability of the h locus (Streisinger & Franklin, 1956).

Further investigations of the host-range locus and r_s mutation have shown that the interaction between the two distant regions of the coliphage T2 genome which produces the star phenotype on coli B,B is even more complicated than has been evident up to this point. It appears on the one hand that to many of the various h^+ and ht states of the host-range locus there may correspond some configuration of the r_s region in which there *still* takes place that interaction between the two regions of the phage genome which results in the star phenotype. For instance, in the presence of the entire r_s mutation, the original wild h^+ allele, unlike secondary h^+ and ht mutations sported by the hr_s star phage, still produces the star phenotype, since about 4% star-type recombinants can be observed among the progeny of the crosses $h_{\text{wild}}^+ r^+ \times h_{s1}^+ r_s$ and $h_{\text{wild}}^+ r^+ \times ht_{s4} r_s$. (The host-range phenotype of these star recombinants is h^+, indicating that their star character does not arise from the reconstitution of h by crossing two non-allelic h^+ loci of independent origin.) The h_{wild}^+ allele, therefore, fails to cause the star phenotype only in phages where it is present in conjunction with parts of the r_s mutation, as in the r recombinants described in Table 2.

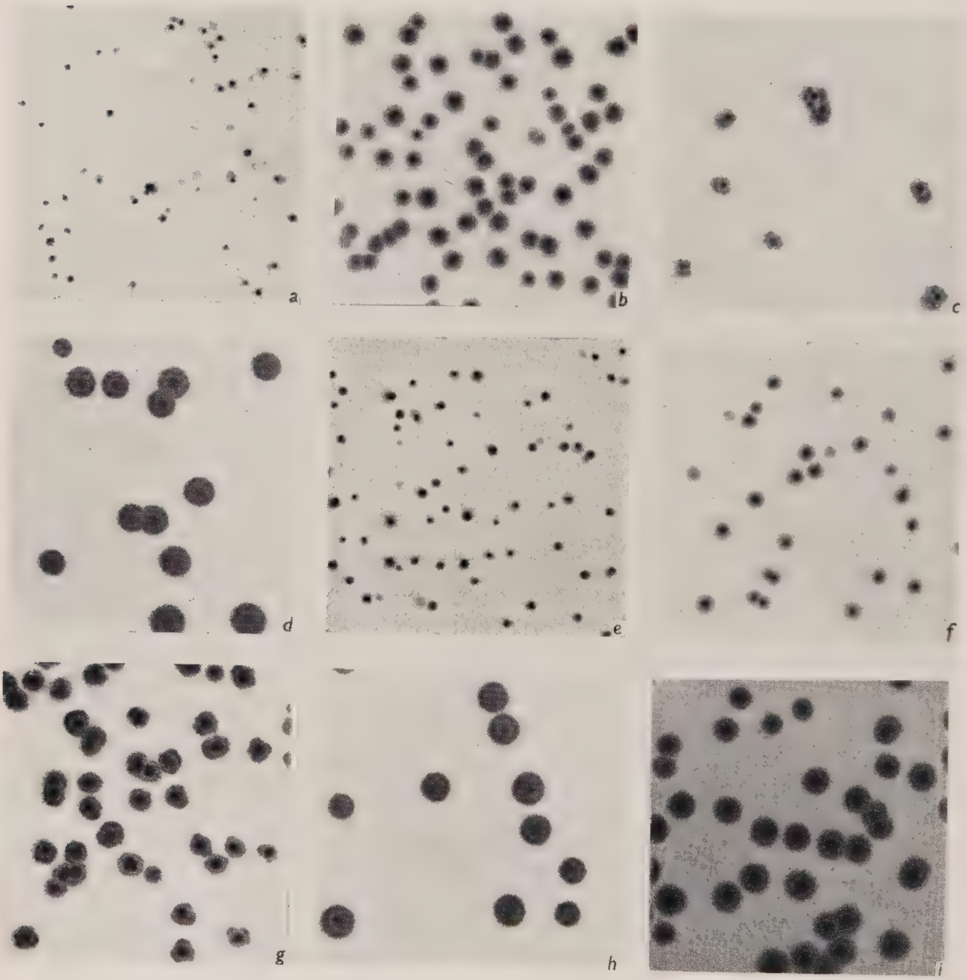
The behaviour of secondary h^+ mutations like h_{s1}^+, on the other hand, is in this respect exactly opposite to that of h_{wild}^+. In experiments, which shall not be presented here in detail, it was found that h_{s1}^+ will endow those phages with the star phenotype which possess only that part of r_s which lies outside the r_{II} region. The star plaques formed by phages harbouring h_{s1}^+ and the non-r_{II} sector of r_s naturally contain once more two types of phages: the parental star types and secondary r mutants. By isolating also such secondary r mutants and carrying out genetic tests similar to those described in the foregoing, it could be demonstrated that these r mutants derive their phenotype from further mutations which modify h_{s1}^+ in such a way that the interaction between the host-range locus and the particular residual sector of the r_s deletion inhibitory to plaque development is once more abolished.

DISCUSSION

The experiments described in the foregoing have endeavoured to characterize the genetic structure of three primary star types and of the secondary mutants into which they dissociate in every plaque. No attempt was made here, however, to determine the physiological causes of the plaque growth inhibition with which these particular star types are afflicted and from which their secondary mutants are released. It is possible that in one or all of the cases discussed here, and as Symonds (1958) could show in the star mutant s_1, the primary star mutation engenders an extension of the normal latent period on host bacteria which have passed out of their exponential phase of growth. Numerous other reasons for the poor plaque development characteristics of star mutants can also be conceived, however, and a definite experimental decision between these possibilities is made rather difficult by the fact that the mechanics of plaque formation and the exact physiological environment in which the growing phage clone finds itself on the surface of an agar plate densely seeded with indicator bacteria are themselves very poorly understood. It is possible, for instance, that star mutant phages may be more sensitive than wild-type or secondary mutants to inactivation by the bacterial debris sure to exist in high concentration in every plaque. Or, one might suppose that the intracellular multiplication of star mutants is slower than that of wild-type, as we have indeed reason to believe in the case of the P^{32}-decay star. It could also be imagined that the poor plaque development is due to the slow adsorption of the star mutants to the indicator bacteria on the agar plate, or even to the appearance of mutant clones among the indicator bacteria resistant to the primary star phage but not to wild-type or to the secondary phage mutants sported by the star phage. All the possibilities mentioned can be reconciled with the observation that the star mutants express their star phenotype only on some indicator strains but that on others, they give rise to normal plaques.

It would seem that any bacteriophage type should form star plaques if it can sport with sufficiently high probability, i.e. soon enough during the development of each plaque, secondary mutants of superior growth characteristics. Such star mutants should be encountered, not only with bacteriophages but with all those plant and animal viruses for which there exist plaque assay methods. In fact, as Terada *et al.* (1956) pointed out, the equivalent of star mutants is also encountered with other micro-organisms which lend themselves to the formation of colonies on solid media. For example, strains of bacteria have long been known among which every cell gives rise to genetically heterogeneous colonies under suitable conditions of plating (Massini, 1907; Müller, 1911; Lewis, 1933; Kristensen, 1944). As in the case of star phages, it can be shown that such bacterial types owe their peculiar colonial morphology and genetic 'dissociation' to some inhibition of colony development of the parental strain and to the appearance and subsequent selection of secondary mutants of superior growth properties.

We wish to thank Martha B. Baylor and Rose M. Litman for supplying us with their star mutant bacteriophage strains, G. Sato and N. Symonds for valuable



E. McFall and G. S. Stent—Star mutants of coliphage. Plate 1

(*Facing p.* 363)

suggestions and discussions, and Margery Hoogs for assistance with some of the experimental work. This research was supported by grants from the National Cancer Institute of the National Institutes of Health, United States Public Health Service, and The Rockefeller Foundation.

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EXPLANATION OF PLATE

The plaque morphology of primary star mutants of coliphage T2 and of recombinants and secondary mutants to which they give rise. (*a*) The 'bromouracil' star mutant s_8r^+. (*b*) The secondary mutant $s_8^+r^+$. (*c*) The 'extended-host-range' star mutant $h'r_1$. (*d*) The secondary mutant $h'i_1r_1$. (*e*) The recombinant $h'r^+$. (*f*) The secondary mutant $h'i_1r^+$. (g) The 'P^{32}-decay' star mutant hr_s. (*h*) The recombinant h^+r_{s1}. (*i*) The secondary mutant $h_{s1}^+r_s$.

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Turbidity Changes in Suspensions of Gram-Positive Bacteria in Relation to Osmotic Pressure

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SUMMARY: The turbidity of suspensions of Gram-negative micro-organisms is known to be influenced by the tonicity of the suspending medium. In contrast to this, suspensions of Gram-positive micro-organisms have the same optical density in 0·2 M-NaCl as in distilled water. At higher salt concentrations, however, occasionally some batches were found to display changes in turbidity. Correlation was shown between the concentration of the amino acid pool of the organisms and their response to changes in osmotic pressure of the suspending medium. Depletion of the amino acid pool resulted in an increase in the changes in turbidity, with the simultaneous suceptibility of Gram-positive bacteria to plasmolysis. A possible correlation between this optical effect and the separation of cytoplasmic membrane from the bacterial cell wall is discussed.

It was reported in preceding communications (Mager, Kuczynski, Schatzberg & Avi-Dor, 1956; Avi-Dor, Kuczynski, Schatzberg & Mager, 1956), that variations in the osmotic pressure of the suspending medium elicited corresponding changes in turbidity of Gram-negative bacteria. The light-scattering ability of suspensions of Gram-positive micro-organisms was found, however, to be independent of the prevailing tonicity. The object of the present study was to investigate the cause of the different response of these two classes of micro-organisms to changes in the osmotic pressure.

METHODS

Culture media. Escherichia coli was grown on nutrient agar (Difco) at 37°. *Bacillus megaterium* was cultivated in a 2% (w/v) solution of an enzymic casein hydrolysate (N.B.C.), fortified with 0·1% (w/v) Marmite and incubated at 30° with shaking. *Staphylococcus aureus* was grown under the same conditions as *B. megaterium* with the addition of 1% (w/v) glucose to the culture medium.

Inoculum. A volume of 0·1 ml. of 24 hr. slope culture of bacteria suspended in 5 ml. saline was used for inoculation of 100 ml. medium.

Duration of incubation: 18 hr.

Chemicals. All chemicals used were of reagent grade.

Determination of ninhydrin-reacting substances released by boiling. A 2 ml.

sample of the bacterial suspension (100 mg. wet weight/ml.) was boiled for 10 min., cooled, centrifuged and 0·2 ml. of the supernatant fluid analysed for ninhydrin-positive substances (Yemm & Cocking, 1955); a similarly treated unboiled sample served for a blank determination. The amount of ninhydrin-positive substances was expressed as μmole glutamate.

Procedure for measuring the optical effect. The optical effect (O.E.) was measured as described before (Mager *et al.* 1956). The magnitude of the effect (E') was expressed as a percentage

$$E' = \frac{E_s - E_w}{E_w} \times 100,$$

where E_w is the light extinction of the suspension in water, and E_s the extinction in the solute.

RESULTS

In a previous communication, Mager *et al.* (1956) reported an increase in the light extinction of Gram-negative bacterial suspensions in isotonic saline or glucose as compared with suspensions in water (optical effect: O.E.). Under these conditions of assay no appreciable change could be demonstrated with Gram-positive bacteria. In further studies, however, when an increased concentration of NaCl was used (0·5 M) occasionally batches of Gram-positive micro-organisms were encountered which showed the optical effect in varying degree. It was also observed that such optically-responsive batches of organisms possessed, as a rule, lower values for the concentration of the amino acid 'pool' than the suspensions which displayed no optical effect. Some representative data on the relation between the content of ninhydrin-positive substances of batches of *Bacillus megaterium* and O.E. values are assembled in Table 1. With *B. megaterium* and other Gram-positive species tested, batches showing more than a 20% O.E. invariably contained lower concentrations of the amino acid pool.

Table 1. *Correlation between optical effect and content of ninhydrin-reacting substances in different batches of* Bacillus megaterium

The amount of ninhydrin-reacting substances released by boiling of the organism was determined as described in 'Methods' and is calculated as 'glutamate'.

Batch no.	E'* value in 0·5 M-NaCl	μmole 'glutamate'/100 mg. (wet weight) bacteria
1	0	4·9
2	5	4·0
3	10	4·2
4	10	4·5
5	20	2·9
6	25	3·3
7	32	2·4

* E' denotes percentage increase in light extinction.

In order to test a possible correlation between the content of ninhydrin-positive substances in the bacteria and the O.E. values, *Bacillus megaterium* was incubated under conditions which caused a depletion of the amino acid pool. Among the media tested (distilled water, phosphate and phosphate + glucose), phosphate buffer (pH 7·3) was found to be the most effective. As seen in Table 2, parallel with the depletion of the amino acid pool the O.E. values increased appreciably. With *Staphylococcus aureus* the addition of glucose to the incubation medium proved to be necessary to lower the content of ninhydrin-positive substances, in accordance with the findings of Gale (1953). The effect of the period of incubation on the changes in the amino acid pool in relation to the O.E. value, was investigated with *S. aureus* (Table 3).

Table 2. *Influence of incubation of* Bacillus megaterium *with phosphate buffer on the optical effect and the content of ninhydrin-reacting substances*

The washed suspension of *Bacillus megaterium*, harvested after 18 hr. growth, was incubated with 0·05 M-phosphate buffer, (pH 7·3) at 28° for 2 hr.

	Before incubation		After incubation	
Batch no.	*E′* value	'Glutamate content' (μmole)	*E′* value	'Glutamate content' (μmole)
1	7	4·5	26	1·6
2	11	4·5	31	1·9
3	12	4·9	26	1·5
4	17	2·4	30	1·5
5	18	2·6	31	1·5

Table 3. *Influence of incubation of* Staphylococcus aureus *with phosphate-glucose medium, on the optical effect and on the content of ninhydrin-reacting substances*

Washed *Staphylococcus aureus* was shaken in a medium containing 0·01 M-phosphate buffer (pH 7·3), 0·01 M-NaCl, 0·15 M-KCl and 0·01 M-glucose at 37°.

Period of incubation (hr.)	*E′* value	'Glutamate' content, μmole/100 mg. wet wt. organisms
0	0	13·8
2	0	10·2
5	0	7·1
24	23	1·6

In previous study on the optical effect in Gram-negative bacteria the osmotic nature of the phenomenon was indicated by the finding that different substances, both electrolytes and non-electrolytes, elicited turbidity changes. Urea, known to be highly diffusible through biological membranes, was ineffective in this respect. With *Bacillus megaterium* (with amino acid pool depleted by incubation with phosphate) only electrolytes elicited the optical effects. A comparison of the effects of the addition of increasing amounts of

NaCl, sucrose, or urea to suspensions of *B. megaterium* and *Escherichia coli* is shown in Fig. 1. As can be seen the optical density of the suspension of *B. megaterium* was increased by the addition of NaCl, remained practically unchanged in urea but in contrast to the response of Gram-negative bacteria it showed a negative O.E. value with sucrose.

Gram-positive bacteria are distinguished by their high internal osmotic pressure, due mainly to the accumulation of amino acids (20–30 atmospheres in *Staphylococcus aureus* in contrast to 5–6 atmospheres in *Escherichia coli*; Mitchell & Moyle, 1956) and also by being refractory to plasmolysis even in concentrated salt solutions (Eisenberg, 1910). It was thought therefore that the depletion of the amino acid pool which made *Bacillus megaterium* optically responsive to changes of osmotic pressure of the medium might render it also susceptible to plasmolysis. Indeed it was found that the characteristic microscopic picture of *B. megaterium* in water suspensions (i.e. the cytoplasm occupying the whole cellular space; see Pl. 1, fig. 1) was changed by the addition of NaCl only in 'depleted' cells showing the optical effect. In this case the shrinkage and the fragmentation of the cytoplasm was noted (Pl. 1,

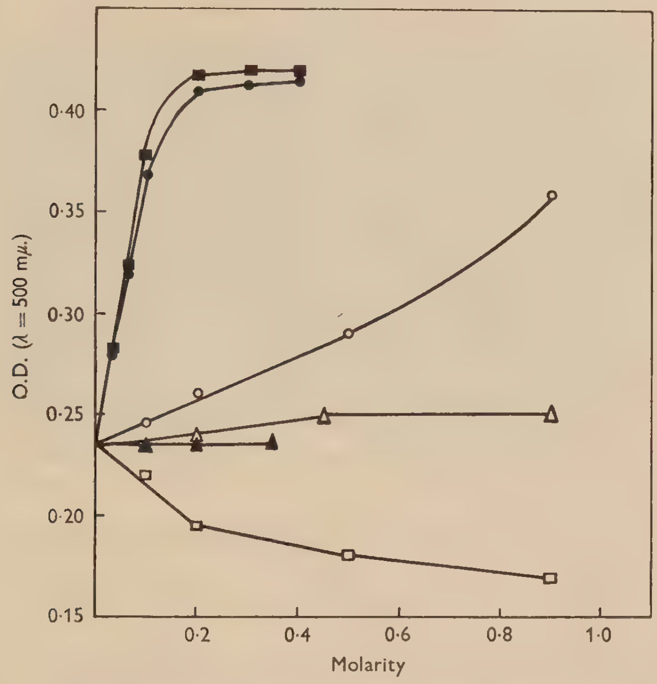

Fig. 1. Effect of solutes on the light extinction of suspensions of *Escherichia coli* and *Bacillus megaterium*. To washed organisms suspended in distilled water, various concentration of NaCl (○—○, ●—●), sucrose (□—□, ■—■) or urea (△—△, ▲—▲) were added, and the light extinction at 500 mμ. measured. The *B. megaterium* was pretreated with phosphate as described in Table 1. Unshaded marks = *B. megaterium*; shaded marks = *E. coli*.

fig. 2). Similar to the increased light scattering, these structural changes were fully reversed by suspension of the bacteria in water. In contrast to the behaviour of the 'depleted' micro-organisms, organisms showing no optical effects remained as in Pl. 1, fig. 1, in spite the addition of salt.

DISCUSSION

In Gram-positive micro-organisms changes in light extinction due to variations in the osmotic pressure were demonstrated only after the amino acid pool of the organisms was depleted by a suitable treatment. The close correlation between the appearance of the optical effect and the susceptibility of the organisms to plasmolysis suggest that the high internal osmotic pressure prevailing in Gram-positive micro-organisms might be responsible for the lack of optical response and the absence of shrinkage of the cytoplasm. With suspensions of mitochondria, turbidity changes due to osmotic pressure were attributed to swelling (Cleland, 1952). Volume changes in the bacterial cytoplasm might be similarly responsible for the optical effect. This was demonstrated in *Bacillus megaterium* by using the light microscope. A similar technique seems to be inadequate for the observation of the phenomenon in the much smaller Gram-negative bacteria.

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EXPLANATION OF PLATE

Fig. 1. *Bacillus megaterium* pretreated with phosphate to deplete amino acid pool (see Table 1); suspended in water.

Fig. 2. The same organisms as in Fig. 1, after addition of NaCl (to final concentration 0·5 M).

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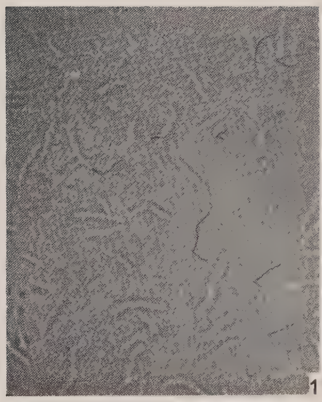

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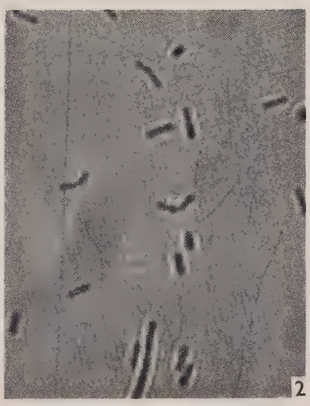

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M. KUCZYNSKI-HALMANN, Y. AVI-DOR AND J. MAGER—TURBIDITY CHANGES IN BACTERIAL SUSPENSIONS. PLATE 1

(*Facing p.* 368)

STEEL, R. & WALKER, T. K. (1958). *J. gen. Microbiol.* 18, 369–376

Studies on the Antibacterial Activity of Certain Strains of *Acetobacter*

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SUMMARY: A turbidimetric assay is described for antibiotic substances produced by *Acetobacter acetigenum*, NCIB 8132, and *Bacterium xylinoides*, NCIB 4940, based on the amount of inhibition of growth of a celluloseless mutant. Yield of antibiotic from each organism was independent of the source of nitrogen in the medium, but varied markedly with the carbon source and was highest when the organisms were grown for 10 days at 30° in a medium containing glucose (9 %, w/v), Difco yeast-extract (0·5 %, w/v) and peptone (Evans, 3 %, w/v), in distilled water. Under these conditions growth of the assay organism was inhibited 50 % by filtrate diluted 1/44. The antibiotics were extremely alkali-labile being destroyed at 18° in 2 min. at pH 10. Activity of the filtrate from organism NCIB 8132 was not affected significantly by treatment with calcium carbonate, charcoal, cation- or anion-exchange resins, but was decreased by extraction at pH 3·0 with *n*-butanol, chloroform or methyl ethyl ketone. Activity of filtrate of organism NCIB 4940 was decreased by treatment with charcoal, cation- or anion-exchange resins and by extraction at pH 3·0 with benzene, *n*-butanol, chloroform, diethyl ether, ethyl acetate or methyl ethyl ketone.

Previous work (Steel & Walker, 1957*a*) on celluloseless mutants (organisms which do not produce extracellular cellulose) revealed that they occurred spontaneously in cellulose-producing cultures of certain *Acetobacter* species. It was later found that four cellulose-forming organisms representing three different species produced factors which inhibited the growth of mutant organisms (Steel & Walker, 1957*b*). Only one culture (*Acetobacter acetigenum*, NCIB 8132) was tested against *Proteus vulgaris* and it was found to be active against this organism. The present work was undertaken (i) to obtain an assay method for these antibiotics, (ii) to assess the effect of variations in the medium on their yield, and (iii) to study some of their properties.

METHODS

Cultures. The cellulose-forming bacteria studied were *Acetobacter acetigenum*, NCIB 8132, and *Bacterium xylinoides*, NCIB 4940. *Proteus vulgaris*, NCIB 67, was tested as assay organism. These cultures were received from the National Collection of Industrial Bacteria, Teddington, Middlesex. The celluloseless mutants tested as assay organisms were obtained by methods described previously (Steel & Walker, 1957*a*). The cellulose-producing organisms were stored in malt-wort medium (Tosic & Walker, 1946), whereas the others were kept on a medium (pH 7·0) containing glucose (3 %, w/v) and Difco yeast-extract (0·5 %, w/v) in distilled water (hereafter, GYE medium). The use of these media was dictated by previous findings (Steel & Walker, 1957*a*).

Media composition. Media (of the composition given below and at pH 7·0 unless stated otherwise) were dispensed either in 150 ml. quantities in mould culture-flasks (Clayton *et al.* 1944) or in 50 ml. portions in 250 ml. conical flasks. After sterilization at 15 lb./sq.in. for 15 min. they were inoculated by a sterile wire loop from a 48 hr. GYE culture of the appropriate organism and incubated at 30°.

Assay procedure. The following was selected as the standard assay method after preliminary experiments, described later, had been made. After passage of the culture medium through a Seitz filter to remove the bacteria, the filtrate was adjusted with sodium hydroxide to pH 6·0 and the desired volume was dispensed in triplicate to nephelometer tubes previously randomized (Brownlee, 1949). The volume was made up to 6 ml. with assay medium (pH 6·0) consisting of glucose (3%, w/v), Difco Casamino acids (0·5%, w/v) and peptone (Evans, 0·5%, w/v) in distilled water. Triplicate tubes of assay medium (6 ml.) containing no added filtrate were included as positive controls. The tubes were plugged with cotton wool, sterilized at 10 lb./sq.in. for 10 min., then inoculated with one drop (*c.* 0·05 ml.) of a 24 hr. culture of a celluloseless mutant, designated as 8132–A 2, grown in the assay medium. After incubation at 30° for 24 hr. the turbidity was measured. These turbidity values were corrected by subtracting the reading given by the uninoculated media (blank). Then the percentage of growth inhibition (the parameter used in this paper to express antibiotic activity) was calculated from the expression

$$\frac{\text{Turbidity of control} - \text{turbidity of test solution}}{\text{Turbidity of control}} \times 100.$$

The resulting value plotted on semi-logarithmic paper against culture dilutions showed a linear relationship (Fig. 1). The estimate of growth obtained in the control cultures gave a standard error among replicates which varied from 0 to 12% (average 5·8%) from one experiment to another. In those instances where the variability among replicates was excessively large, the experiments were repeated. It was realized that the growth of the assay organism in media containing added filtrate might differ from that in control media not containing added filtrate because the media were not nutritionally equivalent, but this difficulty could not be circumvented. In those cases where a significant difference between treatments was not obvious from inspection of the data, the mean turbidity values were compared statistically (Brownlee, 1949).

RESULTS

Preliminary assay experiments. Tests with an active culture filtrate of both organisms showed that sterilization at 10 lb./sq.in. for 15 min. or passage through a Seitz filter or a membrane filter gave products all having the same potency. Clarification by Seitz filtration followed by heat sterilization was selected as the standard procedure for ease of handling a large number of solutions.

The pH during sterilization was found to be critical. At pH 7·0 or higher all activity appeared to be destroyed while media sterilized at pH 5·0 gave slow growth of the assay organism. Hence pH 6·0 was adopted in all subsequent work.

A celluloseless mutant designated 8132–A2 was found preferable as assay organism to the other mutants tested and to *Proteus vulgaris*, since it showed a uniform and slightly faster rate of growth.

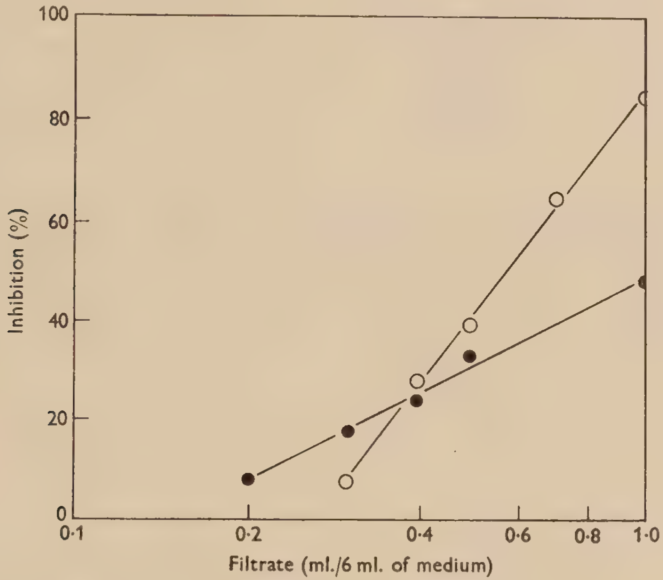

Fig. 1. Dose-response graphs. ●—●, filtrate of *Acetobacter acetigenum*, NCIB 8132, grown in GYE medium; ○—○, filtrate of *Bacterium xylinoides*, NCIB 4940, grown in glucose (6 %, w/v), Oxoid soya-peptone (3 %, w/v) medium; filtrates assayed at 6 days.

Some factors affecting the yield of antibiotics

Nitrogen sources. The following compounds added primarily as sources of nitrogen, although they also contain carbon, were tested singly in a basal medium consisting of glucose (3 %, w/v) in distilled water: Difco yeast-extract (0·5 %, w/v), Oxoid soya-peptone (3 %, w/v), cornsteep liquor (2 %, w/v) and Evans's peptone (3 and 6 %, w/v). Cornsteep liquor and Evans's peptone were also tested in combination with Difco yeast-extract. After 10 days' incubation organisms NCIB 8132 and NCIB 4940 gave essentially similar potencies on all media tested.

Carbon sources. With organism NCIB 8132 in a basal medium consisting of Difco yeast-extract (0·5 %, w/v) and Evans's peptone (2 %, w/v) in distilled water, the supplementary carbon sources listed in Table 1, when added at 3 % (w/v), gave the results shown. Organism NCIB 4940 gave similar results. Other experiments (Table 2) indicated that 9 % (w/v) was the optimum concentration of glucose and that variations in the peptone concentration in media containing 6 or 9 % (w/v) glucose did not affect the yield of antibiotics significantly.

Table 1. *Effect of certain carbon sources on antibiotic production by* Acetobacter acetigenum *NCIB* 8132.

The carbon sources were added to a basal medium (pH 7·0) consisting of Difco yeast-extract (0·5 %, w/v) and Evans's peptone (2 %, w/v) in distilled water. Activity is expressed as a percentage of that of the control; filtrates assayed at 7 days at dilution 1/12.

Growth medium	Relative antibiotic activity (%)
Basal medium (control)	100
+maltose	57
+glycerol	89
+glucose	157
+xylose	164

Table 2. *Effects of concentration of glucose and of peptone on the yield of antibiotic produced by* Acetobacter acetigenum *NCIB* 8132

Glucose and peptone were added to a basal medium (pH 7·0) consisting of Difco yeast-extract (0·5 %, w/v) in distilled water; filtrates assayed at 8 days at dilution 1/20.

Addition to basal medium		
Glucose (%, w/v)	Peptone (%, w/v)	Relative yield of antibiotic
6	3 (control)	100
6	6	104
9	3	133
9	6	136
12	3	111

Effect of calcium carbonate. Neither organism formed detectable antibiotic when grown in a medium containing glucose (6 %, w/v), Difco yeast-extract (0·5 %, w/v) and Evans's peptone (2 %, w/v) in distilled water to which calcium carbonate (2 %, w/v) had been added and the pH adjusted to 6·5. In the same medium, but without calcium carbonate, organisms NCIB 8132 and NCIB 4940 gave 80 % and 67 % inhibition, respectively, at dilution 1/12.

The course of cellulose formation and antibiotic production

Organism NCIB 8132 was grown in flasks of medium (pH 5·8) containing glucose 9 % (w/v), Difco yeast-extract 0·5 % (w/v) and Evans's peptone 3 % (w/v) in distilled water for different periods of time (1–11 days). The cellulose from duplicate flasks was washed in several changes of distilled water and then in methanol, and dried to constant weight at 80°. The filtrates were then assayed and the dilution at which the growth of the assay organism was inhibited by 50 % was obtained. The results are shown graphically in Fig. 2. The inhibitory activity and the dry weight of cellulose increased with incubation time, the former reaching a maximum value (50 % inhibition at dilution 1/44) at about 10 days. The pH of the culture medium decreased to 3·7 in about 6 days and remained constant thereafter. Filtrate from cultures

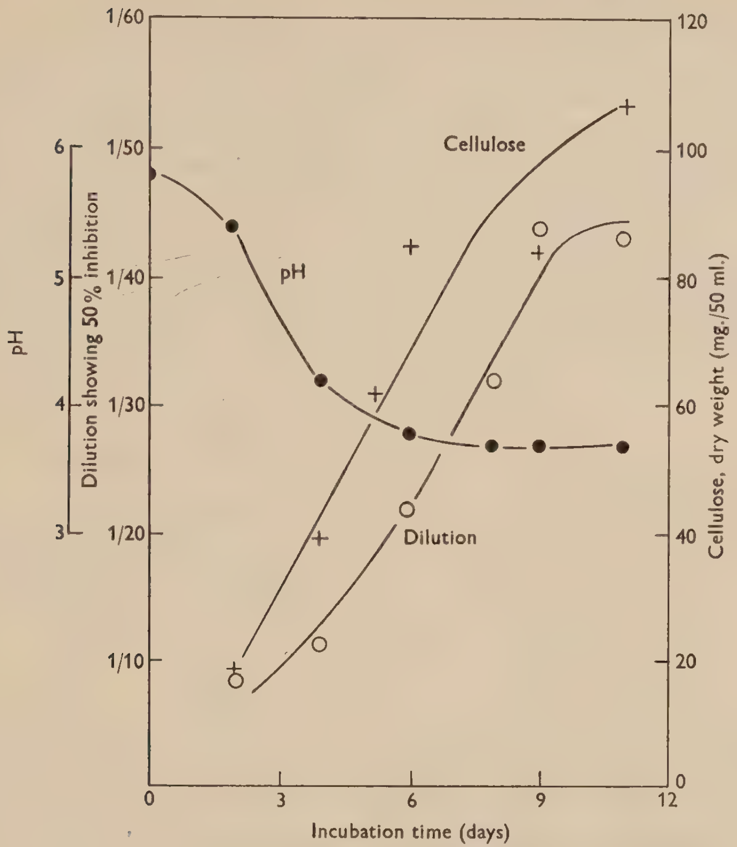

Fig. 2. Biochemical changes during growth of *Acetobacter acetigenum*, NCIB 8132. Growth medium was glucose (9 %, w/v), Difco yeast-extract (0·5 %, w/v) and Evan's peptone, (3 %, w/v) in distilled water.

harvested at 10 days completely inhibited the growth of the assay organism at dilution 1/12 but not at dilution 1/15.

Some properties of the antibiotics

Most of the filtrates used in this part of the work were obtained from 8- to 10-day cultures grown in a medium (pH 7·0) containing glucose (6 %, w/v), Difco yeast-extract (0·5 %, w/v) and Evans's peptone (2 %, w/v), in distilled water.

Stability of the antibiotics at various pH *values.* The activity of filtrates from either test organism showed no significant loss after keeping at room temperature for 24 hr. at pH values of 3·0 or 6·0. At pH 10, however, all activity was lost in 2 min. and, in fact, the filtrate stimulated the growth of the assay organism.

Effect of distillation of filtrate. Fifty ml. of each culture filtrate (pH 6·0) were concentrated *in vacuo* to *c.* 10 ml., then the concentrate was made up to the original volume with distilled water. These solutions showed the same activity as the starting materials.

Effect of adsorbents and ion exchange resins. Samples of filtrate (20 ml., pH 6·0) were shaken with 0·6 g. calcium carbonate and after 30 min. the suspension was centrifuged. Other samples were treated with the same weight of charcoal, but the charcoal was removed by filtration; a preliminary test showed that filtration alone did not affect the activity. Other 20 ml. portions of filtrate were treated with ZeoKarb 225 (H) after which the resin was removed and the filtrate, whose pH had been reduced from 6·0 to 3·5, was adjusted to pH 6·0. Another sample of filtrate was adjusted to pH 3·5 before treatment with Amberlite IR–4B (OH), during which the pH rose to 6·0.

Table 3. *Effect of certain adsorbents and ion-exchange resins on the inhibitory activity of culture filtrates*

Residual activity is expressed as a percentage of the value obtained with untreated filtrate assayed at dilution 1/12.

	Activity of filtrate of	
Filtrate treatment	*Acetobacter acetigenum*, NCIB 8132	*Bacterium xylinoides*, NCIB 4940
None (untreated)	100	100
Calcium carbonate	100	81
Charcoal	109	13*
Zeo-Karb 225 (H) (cation)	102	54*
Amberlite IR-4B (OH) (anion)	79	45*

* Differs from the value for untreated filtrate at the 5 % level of significance.

Table 4. *Effect of solvent extraction of culture filtrates on inhibitory activity*

Residual activity is expressed as a percentage of that of untreated filtrate assayed at dilution 1/12.

	Residual activity of filtrate of	
Solvent	*Acetobacter acetigenum*, NCIB 8132	*Bacterium xylinoides*, NCIB 4940
None (untreated)	100	100
Benzene	120	24*
n-Butanol	70*	60*
Chloroform	71*	48*
Diethyl ether	100	26*
Ethyl acetate	95	54*
Methyl ethyl ketone	60*	42*
Methyl *iso*-butyl ketone	91	—

* Differs from the value for untreated filtrate at the 5 % level of significance.

The results (Table 3) indicate that the inhibitory agent from organism NCIB 8132 was not significantly adsorbed, whereas that of organism NCIB 4940 was removed partially with charcoal and ion-exchange resins, but not by treatment with calcium carbonate.

Solvent extraction. Samples of filtrate (pH 3·0) were shaken in a separatory funnel with an equal volume of the selected solvent. The aqueous phase was freed from solvent by vacuum distillation, adjusted to pH 6·0, made up to the original volume with distilled water and assayed. The results (Table 4) show that in this respect, too, the antibacterial activity from the two organisms behaves differently. In other experiments the antibiotic from organism NCIB 8132 was not extracted at pH 6·0 with benzene, *n*-butanol, chloroform or ethyl acetate.

Effect of proteolytic enzyme. When 1 ml. of a 2% (w/v) solution of Evans's Pepsinum in 0·001 N-hydrochloric acid was added to 10 ml. samples of filtrate (pH 3·0) and the mixtures incubated at 30° for 24 hr., the titres remained the same as that of a control made up with boiled enzyme. Experiments with gelatin showed that the enzyme preparation was active.

DISCUSSION

In previous studies (Steel & Walker, 1957*b*) with organism NCIB 8132 it was found that filtrate obtained from cultures grown in GYE medium inhibited the growth of the assay organism by 50% at dilution 1/3. In the present work the highest filtrate dilution producing a similar amount of inhibition was about 1/44 (Fig. 2), indicating an increase in yield of about 15-fold.

The antibiotic yield from both organisms (NCIB 8132 and NCIB 4940) was independent of the nitrogen source but was influenced by the type and amount of the carbon source available. Both antibiotics were extremely labile to alkali. That the two inhibitors were not identical was indicated by the difference in slopes of the assay plots shown in Fig. 1 (although these are not strictly comparable because different media were used), and by differences in behaviour towards adsorbents and solvents (Tables 3 and 4).

The failure of cultures containing calcium carbonate to produce antibiotics was unexpected. This might have been caused by the diversion of carbohydrate metabolism away from the direction of the antibiotic towards the formation of calcium salts of organic acids, e.g. gluconic, ketogluconic (see Kulka & Walker, 1954) or perhaps the antibiotic was adsorbed on the calcium carbonate during the rather long period of incubation (8 days). Further study of these substances is planned.

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Ramycin: A New Antibiotic

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SUMMARY: A strain of *Mucor ramannianus* from Campine forest soil was found to secrete an antibiotic, for which the name Ramycin is proposed. It is especially active against Gram-positive micro-organisms and *Myobacterium tuberculosis* H37Rv, but sensitive organisms quickly acquire resistance to it. Although it is not very toxic, it possesses a necrotic action on tissues. Ramycin can protect mice against streptococcal and staphylococcal infections but not against H37Rv.

Ramycin crystallizes in orthorhombic plates. The new antibiotic is an optically-inactive unsaturated hydroxy acid, containing C, H and O only.

In general, it is considered that the Phycomycetes build up relatively simple metabolic products only and that they are not capable of synthesizing the complex molecules characteristic of so many antibiotics (Brian, 1951). In fact, the only reports of antibiotic activity from this group which we have found are those of Jefferys, Brian, Hemming & Lowe (1953) and of Miller & Porter (1956–7). The last authors reported that the action was due to common organic acids such as fumaric and lactic acid.

In a study of the microbial population of soils from Campine in Northern Belgium we isolated a strain of *Mucor ramannianus* which forms a new antibiotic. This has been isolated in crystalline form and the name Ramycin is proposed for it. This report presents some biological and chemical characteristics of this new product.

RESULTS

Cultural characteristics of the fungus

On potato agar (potato scrapings 200 g., glucose 10 g., agar 15 g., tap water 1 l.) the fungus grows as an interlacing network of white sterile filaments lying loosely on the surface of the medium, with a pink fluff of aerial hyphae never more than 3 mm. high. The underside of the mycelium remains uncoloured. On media predominantly mineral, the pink colour is replaced by white and the colony becomes more filamentous through the overgrowth of sterile filaments.

The hyphae are not septate, except some branches of older cultures, especially where sporangiophores arise. The latter vary in length and the sporangia vary in size. Microscopically they appear as brown spheres with spores moving inside them. The columella, which is not visible until after the disruption of the sporangium wall at maturity, is round, and sometimes surrounded by a short collar, which is a remnant of the sporangium wall. The spores are round and very uniform in size, of diameter 1·5 to 2 μ. Rhizoids are absent. Chlamydospores are present but zygospores most probably are not formed. Sometimes we have encountered oval or round spheres surrounded by three or four appendages, yet we have never seen suspensors. The mycelium seems to be homothallic. In submerged cultures the hyphae form rounded buds of irregular size, in yeast-like fashion.

The foregoing properties correspond to those of *Mucor ramannianus* (Naumov, Buchet & Mouraviev, 1939). Robbins *et al.* (1945) found no antibiotic production in this species and we have often isolated antibiotically inactive *M. ramannianus*. We propose the varietal name 'keerbergensis' after the village where the soil sample was collected.

Production of antibiotic

The fungus was maintained on the above-mentioned potato agar. An inoculum was grown at 26°–28° in rotary shakers on the following medium: corn-steep solids 3 g., skim milk powder 3 g., dried brewer's yeast 3 g., NaCl 5 g., glucose 20 g., tap water 1 l., pH adjusted to 7 with 10% NaOH. After 24 hr. it was transferred to the same medium but containing lactose instead of glucose, in either rotary shakers or fermentation tanks. In rotary shakers at 27°–28°, the maximum antibiotic yield (up to 120μg./ml.) was obtained after 52 hr. After this the antibiotic activity decreased, and autolysis of the mycelium started at 80 hr. The pH fell at the beginning and rose after about 10 hr.; later it fell again. We did not observe a correlation between antibiotic yield and sugar utilization, soluble organic-N, mycelium weight or pH. As in the penicillin fermentation lactose, which is less readily metabolized than glucose, gives higher yields of antibiotic. Starch also gives satisfactory yields. To obtain a homogenous growth on rotary shakers we had to inoculate with a heavy spore suspension and use a large quantity of antifoam. In fermentation tanks growth was more homogenous, probably due to the agitation of the impellors.

Extraction

The substance is acidic and can be extracted and concentrated by solvent transfer in water-immiscible solvents. The culture fluid was adjusted to pH 7·5 and filtered on Buchner funnels through Whatman no. 1 filter-paper, covered with a thin layer of infusorial earth. One fifth of its volume of benzene or toluene was added to the filtrate, whose pH was adjusted to 3 and the mixture was shaken. Practically all the Ramycin passed into the solvent, from which it could be re-extracted into a smaller volume of water brought to a final pH of 8. This was adjusted to pH 3 and the product extracted again into ether or chloroform. The extract was concentrated *in vacuo* to about 1% of the original volume. Ramycin was then precipitated by five volumes of petroleum ether and separated.

Chemical and physical properties

The colourless orthorhombic plates in which Ramycin crystallized melted at 158°–160° (decomp.). In the acidic form the antibiotic was very soluble in most organic solvents with the exception of light petroleum. In water at room temperature, about 100μg./ml. dissolved. The antibiotic was optically inactive (1% in ethanol). No specific absorption in the ultraviolet was found.

The infrared spectrum exhibited peaks at 3435, 1727, 1688, 1439, 1380, 1258, 1032 and 973 mμ. The absorption at 3435 indicates probably an alcoholic OH. The titration curve indicates a single acidic group (pK 4·6) and an equivalent weight of 478.

The stability of Ramycin depends principally on the temperature. After 3 min. boiling at pH 2, 70% activity was destroyed, at pH 6, 20% and at pH 8, 80%. No loss of activity was noted after 24 hr. at 37° at pH 2 or 9·5.

The following reactions on active functions were negative: the reactions for amines, amides (boiling with 10% NaOH); the reaction for aldehydes and carbonyl function (Schiff test, reduction of $AgNO_3$, temperature elevation with phenylhydrazine, reaction with *o*-dianisidine); the reaction for sugars (tests with α-naphtol, anthrone and resorcinol); the reaction for phenol having a free OH in ortho position (Millon's test); the reaction for phenol and enol (no colour with $FeCl_3$); the reaction for pyrrol (test with *p*-aminobenzaldehyde); Fehling's solution was not reduced; the biuret reaction was negative.

Positive results were obtained for the following functions: the reaction for CH_3-CHOH-R or CH_3-COR (iodoform test), the test for acids (with Na_2CO_3) and hydroxy acids (with *s*-diphenylcarbazide); test for double bound (bromine fixation in CCl_4). From these tests we may conclude that Ramycin is a hydroxy acid with one or more double bounds.

Ramycin contains only C, H and O, and yields the following data on elementary analysis: C, 69·7%; H, 9·32%; O, 19·53%. Since S is absent and there is no colour with $FeCl_3$ we suppose the acidity is due to a carboxyl group.

Antibacterial action

The sensitivity of various organisms is listed in Table 1. Ramycin is especially active against Gram-positive micro-organisms and against *Mycobacterium tuberculosis* H37Rv. Micrococci are more sensitive than streptococci.

Table 1. *Inhibitory concentrations of Ramycin against different micro-organisms*

Organism	Minimum inhibitory concentration of Ramycin (μg./ml.)
Streptococcus ATCC 8668	1
Micrococcus pyogenes aureus ATCC 6538P	0·05
M. pyogenes aureus resistant to penicillin, streptomycin, tetracyclines, erythromycin and chloramphenicol	0·05
Sarcina lutea	0·05
Salmonella typhi	>500
Brucella abortus	500
Corynebacterium diphtheriae ATCC 9059	0·5
Bacillus mycoides	2
Mycobacterium tuberculosis H37Rv	0·1
M. tuberculosis (clinical case)	0·3
M. lacticola	100
Candida albicans	>500

The organisms were streaked out from 24 hr. old cultures, upon the surface of Trypticase Soy agar (Baltimore Biological Laboratory) wherein the antibiotic was incorporated at 45°. The inhibition was noted after 24 hr. incubation at 37°. The mycobacteria were tested in Dubos medium (Difco) and results read after 7 days at 37°.

We did not find cross resistance to streptomycin, penicillin, the tetracyclines, bacitracin, erythromycin, novobiocin or Staphylomycin (a trade name for antibiotic number 899, De Somer & Van Dijck, 1955). Sensitive micrococci quickly acquired resistance to Ramycin, in a pattern similar to that shown with streptomycin. The antibiotic is not active against poliomyelitis virus type I, II or III.

The activity of the product is not influenced by variation of the pH between 6·5 and 8.

Ramycin is reversibly inactivated *in vitro* by blood serum. When tested in media containing different amounts of bovine serum ranging from 5 to 50 %, 0·5 ml. serum could inactivate 1·4μg. Ramycin. The activity could be recovered again by extraction with organic solvents.

The action of Ramycin is only bacteriostatic. Synergism is observed in combination with staphylomycin. The antibiotics in common clinical use (penicillin, the tetracyclines, bacitracin, streptomycin, chloramphenicol and erythromycin) exhibit only an additive effect against micrococci; similar results were obtained against H37Rv when combined with streptomycin, *p*-aminosalicylic acid and isonicotinic acid hydrazide.

Toxicity. The acute LD 50 for intraperitoneal administration to mice was found to be 165 mg./kg. The animals die 3–4 days after the injection of the Ramycin, without distinct symptoms. Autopsy and histological examination of the organs revealed no abnormalities.

For studying chronic toxicity we injected white mice on 10 consecutive days with 1 mg. intraperitoneally. No important deviations were found in the weight curve or in the blood picture, apart from an increase of 12·9 % in the leucocyte count over that in the control animals. Histological examination showed no lesions in the liver, spleen, brain, kidneys or intestine. Intradermal injections of 500μg. into rabbits caused a little topical necrosis of about 1 mm. diameter, which disappeared after 3–4 days. However, a red irritation zone such as formed by other irritating products was not seen. A solution of 1 mg./ml. in physiological saline caused no irritation to the eye of rabbits.

An intramuscular injection in the thigh of guinea-pigs caused local oedema, subsequently involving the entire limb and leading to progressive necrosis. The administration of Ramycin by this route s thus excluded.

Animal protection tests

The effect of Ramycin given by various routes on mice infected with *Streptococcus pyogenes* or with *Staphylococcus aureus* Strain 663 (kindly supplied by Glaxo Laboratories Ltd.) is summarized in Table 2. Subcutaneous and oral administration gave the best results. However, a survival of 100 % was never attained.

The activity *in vivo* against H37Rv was tested in guinea-pigs, which were infected subcutaneously over the sternum with 0·1 ml. of a 5-day-old culture on Dubos medium. The treatment was started 10 days after the infection and continued for a month after which the animals were killed.

Table 3 summarizes the autopsy findings in treated and control animals.

Table 2. *Chemotherapeutic effectiveness of Ramycin against experimental infections in mice*

	Route of administration				
	Intra-peritoneal	Intra-muscular	Oral	Sub-cutaneous	Not treated
Against *Streptococcus pyogenes* ATCC 8668					
Survival time (hr.)	74	74·4	85	90·7	28·2
Survivals % after 10 days	0	0	20	20	0
Against *Staphylococcus aureus* strain 663					
Survival time (hr.)	49	56	90·8	72	20
Survivals % after 10 days	0	0	10	40	0

The dosage of Ramycin was 1 mg. at 6 and 18 hr. after infection. Groups of 15 animals were used for each treatment. They were infected intraperitoneally with about 10^8 organisms.

Table 3. *Macroscopic involvement of the organs of guinea-pigs infected with* Mycobacterium tuberculosis *strain* H37Rv *and treated with Ramycin*

Daily treatment	Lungs	Spleen	Liver	Axillary lymph nodes	Abcess
10 mg./kg. *per os*	+	+++	+++	⊥	+
50 mg./kg. *per os*	+	++	++	⊥	⊥
10 mg./kg. subcutaneously	⊥	+	+	⊥	+
50 mg./kg. subcutaneously (killed after 15 days)	—	⊥	—	—	+
Untreated	+	+++	+++	+	+

The symbols represent the extent of the tuberculous lesions seen at necropsy (summarized) after 1 months treatment: ⊥: rare or questionable foci of involvement; +: moderate number of tubercles; ++: many tubercles; +++: extensive involvement, nearly confluent tubercles; —: no pathogenic involvement.

It is obvious that Ramycin did not influence the evolution of tuberculosis in these animals when oral doses of 50 mg./kg. were given. When 10 mg./kg. were administrated subcutaneously, a partial protection was noted, but the antibiotic could hardly be considered active *in vivo*. In control experiments using streptomycin (10 mg./kg.) no lesions were found and the weight curve increased regularly.

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An Outline of the Pattern of Bacterial Generation Times

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SUMMARY: The generation times of four species of organisms have been measured, each under several sets of conditions: *Aerobacter cloacae, Serratia marcescens, Streptococcus faecalis* and *Pseudomonas aeruginosa*. Minor variations in the experimental conditions appear to affect the mean generation time less in large samples than in small. This can be explained as a result of association between the generation times of closely related organisms. Positive correlation between the generation times of sisters, cousins and perhaps second cousins shows that the influence of an ancestor is felt through two or three generations. The observed correlation between mothers and daughters is usually small, probably because of bias due to the interval between fission of cytoplasm and fission of cell wall. The coefficient of variation of generation time is not a constant for the species but it is stable under given circumstances. It is possibly related systematically to the chemical complexity of the growth medium. In unhampered growth, less than 1% of the organisms produced are non-viable. There is positive association between the viabilities of sisters, and between the viability of an organism and the generation time of its mother. The distribution of generation times can be represented by a Pearson Type III or else a Pearson Type V distribution; both are convenient in applications.

The generation time of an individual is considered to be determined partly by molecular accidents, partly by heredity.

In a previous paper on this subject (Powell, 1955), I gave an account of some preliminary work designed to test and compare the hypotheses of Rahn (1932) and Kendall (1948, 1952) about the causes of variability in the generation times (τ) of individual bacteria. It turned out that a comparison of the observed distributions of τ with those predicted by the hypotheses was not altogether satisfactory, and I suggested that a further test might be made by determining the effect of alterations in temperature and growth medium on the coefficient of variation of τ. At the same time I concluded that such experiments should await improvements in observational technique. There appeared to be a possibility that the relevance of the observations was altogether vitiated by ‘delayed fission’, that is, by the lapse of an appreciable and variable interval between the termination of the processes considered by Rahn and Kendall, and the overt occurrence of fission.

The results which I now present consist of generation time measurements on unicellular organisms of four species—*Aerobacter cloacae, Serratia marcescens, Streptococcus faecalis* and *Pseudomonas aeruginosa*—each under several different growth conditions. The undertaking was originally encouraged by the development of an improved culture chamber (Powell, 1956*a*), which would permit much greater accuracy in the determination of times of fission. At first, I hoped that the results would be of enhanced diacritical value, but it soon became clear that the generation times of bacteria do not depend on chance in the simple way implied by the hypotheses of Rahn and Kendall. The τ in any one population are interrelated in a complex manner, and in the present state of knowledge can fairly be said to be useless for the purpose of establishing the truth of these or any other analogous hypotheses. So far from losing in interest, the results offer new mathematical and biological problems.

My conclusions, though reached by way of a good deal of arithmetic, are almost entirely of a descriptive character. It would be inappropriate at the moment to attempt any quantitative treatment of the behaviour of a given species under given conditions, and I have presented a broad picture as if it were quite generally true. I believe that it is in fact true of many species under a wide range of growth conditions, but I have no doubt that each several inference will be demonstrably falsified in some instances.

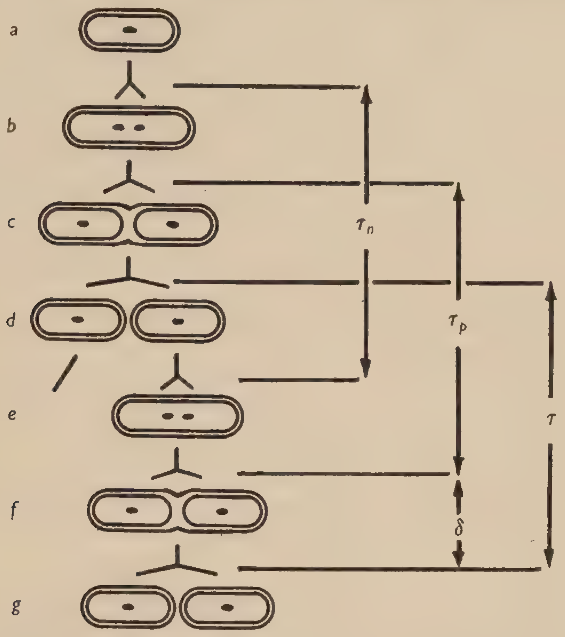

Fig. 1. $a \ldots g$: stages in fission of a unicellular organism. τ_n, τ_p, τ: generation times for nucleus, cytoplasm and cell wall respectively. δ: interval between cytoplasm fission and cell-wall fission.

I have so far used the expression 'generation time' in its ordinary sense—a vague one—and before proceeding to a description of the experiments and the analysis of data, I wish to give it a more precise meaning. The fission of a unicellular organism can be imagined to take place in three obvious stages; these are represented in Fig. 1. At (*a*) a single organism is shown as bounded by the cell wall (outer oval); within it the cell membrane (inner oval) encloses the cytoplasm, and the nucleus appears as a central dot. It is evident that when fission occurs, first the nucleus (*b*), then the cytoplasm (*c*), then the cell wall (*d*), must divide. Only at (*d*) will two mechanically independent particles be produced, though it is likely that the two protoplasts at (*c*) are organically autonomous. These stages are repeated in the first generation progeny (*e, f, g*; only one daughter cell is shown in Fig. 1), and they give rise to three 'generation times': τ_n, τ_p, τ, the intervals between successive fissions of nuclei, cytoplasm, and cell wall, respectively. The morphology and behaviour of the bacterial nucleus is still a matter of controversy. Fission of the nucleus has never been observed; it is not known to be of the unitary character that Fig. 1 suggests, nor is it known that the genetic copying mechanism is carried solely by the nucleus. Accordingly I exclude τ_n from further consideration. The interval τ is that which is measured in the present experiments, i.e. the experimentally determined times of fission correspond to separation into mechanically independent pieces. Where necessary, I distinguish the events terminating intervals τ_p and τ as '*p*-fission' and '*o*-fission' respectively; otherwise, 'fission', 'inception', 'termination' and 'generation time' are to be understood to refer to, or be determined by, *o*-fission. (Most cells are invested with at least a thin mucous capsule, and a pair of sisters may remain attached thereby after the completion of fission. But the capsule has probably no vital function, and such a pair can be separated without damage by means of a micromanipulator. The capsule does not interfere with the observation of fission in the foregoing sense.) The interval δ (Fig. 1) between a *p*-fission and the succeeding *o*-fission is accessible, though with difficulty, to observation, and in certain cases it can be shown that *p*-fission is considerably the earlier (Powell, 1956*a*), though no quantitative work has yet been done. It is likely that δ is always of appreciable magnitude and variance, and I think therefore that Rahn's expression 'delayed fission' as applied to normal cells should not be perpetuated, since it suggests abnormality.

Under the action of certain drugs (e.g. *m*-cresol, acridine dyestuffs, excess of magnesium, radioactivity) most Gram-negative organisms will grow into long filaments which have little or no obvious structure unless they are stained (Pl. 1, *a*). When the filaments are treated with a hypertonic saline solution, the cytoplasm shrinks and can then be seen to be divided into a number of short pieces (Pl. 1, *b*); apparently *o*-fission has been inhibited, but *p*-fission proceeds at nearly the normal rate. Unless the saline treatment is prolonged, the filaments will recover their apparent continuity and continue to grow when the original medium is replaced. When growth is resumed in the absence of the inhibitor, the filaments will at the same time fairly quickly break down into units of normal length, and some at least of the points of fission correspond to gaps which have appeared under the action of saline. Sometimes a portion of a filament will collapse, lose its optical contrast, and grow no further. This is another indication of the existence of independent protoplasts within the elongated cell wall. It seems therefore that *o*-fission is a phenomenon of secondary importance in growth; certainly it ought not to be too readily assumed to be linked directly with fundamental rate-controlling processes. We can expect only a rough correspondence between the two generation times τ_p and τ.

DESCRIPTION OF THE EXPERIMENTS

Method

The organisms were grown under the microscope in an improved culture chamber (Powell, 1956*a*), which permitted observation at a magnification of ×1500 with transmitted phase-contrast illumination. The medium was constantly circulated through the chamber. The experiments were nearly all conducted in the same way: the chamber was inoculated from an overnight culture of the organism to be examined, and left for a period equal to 5 or 6 mean generation times, enough to establish steady growth and a stable age-distribution. (This period, if not known roughly from experience, was estimated in a pilot experiment.) By this time small colonies had been formed; the cover-glass of the chamber was lifted and replaced so as to break them up. Then a field containing a few well-separated single organisms was selected and left for a further 15–30 min. before observation was begun.

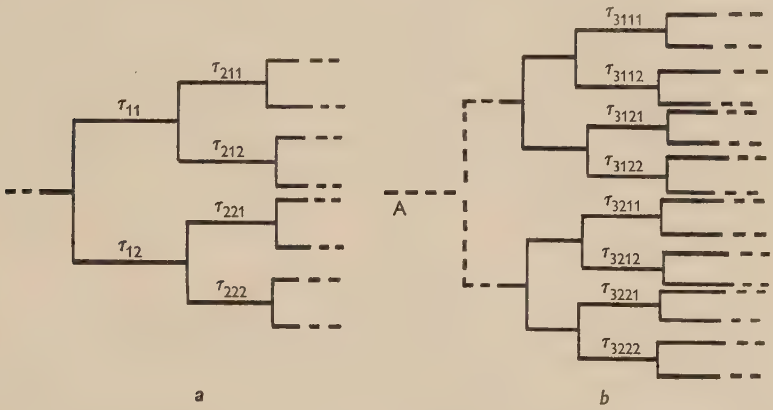

Fig. 2. Typical family trees. The complete horizontal segments represent measured generation times.

The growth of the selected organisms was followed for two complete generations so that each yielded a set of six generation times (two daughters and four granddaughters); Fig. 2*a* shows the typical family tree. The field was watched continuously at critical periods so that the actual times of fission (that is, of separation into apparently discrete pieces) could be estimated; previously the observations had been made at fixed and regular intervals. The generation times, i.e. the intervals between successive fissions, were recorded to the nearest 2 min. or 5 min., according as the average τ was below or above 50 min. The τ values quoted are the means for these groups. With the new optical system, I am confident that few generation times have been assigned to the wrong group as a result of errors of judgement.

During the waiting period after the disturbance of the cover-glass, it frequently happened that some of the selected cells divided. In such cases both daughter cells were followed for two further generations. Each pair thus provided twelve generation times, in two sets of six known to be related as in Fig. 2*b*, though the termination of the original ancestor *A* was not observed.

For conciseness, it is convenient to have a special terminology for sets of organisms (or their generation times) which are related in a particular way. I adopt the following: a '*H*-triad' is a mother and two daughters such as τ_{11}, τ_{211}, τ_{212} in Fig. 2*a*; A '*F*-hexad' is a family of six related as are τ_{11}, τ_{12}, τ_{211}, τ_{212}, τ_{221}, τ_{222}; a '*S*-dyad' is a pair of sisters such as τ_{11}, τ_{12}; a 'C_1-tetrad' is a set of first cousins such as τ_{2ab} $(a, b=1, 2)$; and a 'C_2-octad' is a set of second cousins such as τ_{3abc} $(a, b, c=1, 2)$ in Fig. 2*b*.

In a few experiments, especially where the generation times were very long, observation was continued for one generation only; and in all there were some incomplete sets resulting from the occurrence of non-viable organisms and the escape of motile organisms from the field of view. Each series of observations carried out on a given species under given cultural conditions was continued until a sample of 300–450 individual generation times had been accumulated.

Organisms which have been under observation with phase-contrast illumination in the new chamber can at any time be stained *in situ* without appreciable disturbance. This feature was taken advantage of in some ancillary experiments to determine the morphological status of fission as seen under phase-contrast illumination. Organisms at different stages of development were treated with specific cell-wall stains (see, for example, Bisset, 1955; Tomcsik & Grace 1955); it was found that the time of separation into discrete pieces corresponded approximately to the cell-wall fission—that is, to *o*-fission, stage (*d*) of Fig. 1.

Organisms and media

The following is a list of the growth media used in the experiments:

Media TMB and PW. Conventional tryptic meat broth and peptone water.

Medium H. Devised by Dr D. Herbert (private communication). The medium is made up from three solutions which are stored separately at 0° in Pyrex bottles.

Solution (i): $NaH_2PO_4.2H_2O$, 31 g.; $(NH_4)_2HPO_4$, 238 g.; K_2SO_4, 70 g.; water to 1 l.

Solution (ii): MgO, 4·00 g.; $CaCO_3$, 2·00 g.; $FeSO_4.7H_2O$, 0·56 g.; $ZnSO_4.7H_2O$, 0·29 g.; $MnSO_4.4H_2O$, 0·22 g.; $CuSO_4.5H_2O$, 0·05 g.; concentrated HCl solution 25 ml.; water to 1 l.

Solution (iii): Glucose, 20 g., water to 100 ml.

'Analar' chemicals and demineralized water were used for making up the solutions: batches of the complete medium were freshly prepared as required by mixing: solution (i) 50 ml.; solution (ii) 5 ml.; solution (iii) 50 ml.; water 895 ml.

Medium HP. Medium H with the addition of 0·1 % (w/v) recrystallized sodium pyruvate, introduced as the solid immediately before use.

Medium HG. Medium H with the addition of 0·15 % (w/v) L-glutamic acid.

Medium HT. Medium H with the addition of 0·05 % (w/v) sodium thiosulphate pentahydrate.

Medium HGST. Medium H with the addition of 0·1 % (w/v) succinic acid, 0·15 % (w/v) L-glutamic acid, 0·05 % (w/v) sodium thiosulphate pentahydrate.

It was found necessary to pay great attention to general cleanliness and to the purity and freshness of the chemically defined media, if steady and reproducible growth was to be achieved.

It will be seen that medium H contains glucose, ammonia and sulphate as the sole sources of carbon, nitrogen and sulphur, respectively; the various additions made offer these elements in alternative and for some purposes more readily assimilable forms.

The sixteen series of experiments are set out in Table 1, where they are each given a symbol for convenient reference in later tables. The column headed 'oxygen supply' refers to the gas used to drive the medium-circulating pump, ultimately the source of the oxygen required by the organisms for respiration and growth. Series Ba 2, 3 and 4 each comprise two sets of experiments. In one set the pump was driven by pure oxygen, so that the concentration of dissolved gas was increased. A statistical test showed that neither the generation time nor its variance was significantly altered thereby; thus there was no evidence of inadequate oxygenation, and the two sets were combined. Series Sf 3 is treated separately because the higher oxygen concentration increased both the generation time and its variance very significantly (Powell, 1956*a*). The inocula in series Ba 1, 2 and 3; Cp 1; Sf 1, 2 and 3; Pp 1 and 2, were drawn from cultures maintained on the same media as were used in the experiments themselves; the numerical results for these series probably represent the stable behaviour of the species concerned. This is not necessarily true of the other series. The strain of *Serratia marcescens* chosen was fully chromogenic when grown on complex media, but began to throw off colourless variants after about ten transfers in defined media. In series Cp 2, 3, 4 and 5, therefore,

Table 1. *Organisms and growth conditions*

Series reference symbol	Organism	Medium	Temperature (° C.)	Oxygen supply	No. of experiments
Ba 1	*Aerobacter cloacae*	TMB	30	Air	10
2		TMB	35	Air, O_2	9
3		H	35	Air, O_2	12
4		HP	35	Air, O_2	9
5		HG	35	Air	9
6		HT	35	Air	9
Cp 1	*Serratia marcescens*	TMB	35	Air	9
2		H	35	Air	9
3		HP	35	Air	9
4		HG	35	Air	9
5		HT	35	Air	8
Sf 1	*Streptococcus faecalis*	TMB	30	Air	9
2		TMB	35	Air	11
3		TMB	35	O_2	9
Pp 1	*Pseudomonas aeruginosa*	PW	35	Air	11
2		HSGT	35	Air	10

a mother culture was maintained in tryptic meat broth; inocula from it were subcultured thrice only in medium H before use. In series Ba 4, 5 and 6 the culture was maintained in medium H; it is to be noted, then, that in these three series and in Cp 3, 4 and 5 the organisms under observation were in contact with the fortified media HP, HG and HT for the the first time. *Pseudomonas aeruginosa* grew slowly and uncertainly on the synthetic media with or without a single addendum; medium HSGT, with three, supported rapid growth. There appears to be no easily prepared defined medium suitable for *Streptococcus faecalis*.

The uniformity of Cellophane

The general conclusions of this paper depend on a single ostensible fact, that organisms closely related by descent tend to have similar generation times. Now since such organisms lie in close juxtaposition on the Cellophane (Messrs British Cellophane Ltd.) membrane of the culture chamber, the similarity could conceivably be a mere casual result of inequalities in the permeability of the membrane; organisms overlying a relatively impermeable patch would be poorly nourished. I had therefore originally planned an extensive series of tests for uniformity. However, during the course of the investigations different experiments, both of my own and of independent workers, showed that other properties of bacteria were interrelated in a similar way to the generation times. I think now that the following considerations are sufficient rebuttal of any doubts on this score:

(i) Under the phase-contrast microscope, Cellophane is very uniform in its apparent optical density, except that it contains a great number of well-defined ellipsoidal inclusions, nearly all less than 5μ. across. These are too small, too frequent and too uniformly disposed to be a cause of impermeability on the required scale (they are not of course necessarily impermeable themselves). Fields showing large inclusions near to the surface are naturally avoided in experimental work (Pl. 1, *c*).

(ii) The polarizing microscope also shows no evidence of phase changes such as might be expected in areas of high density.

(iii) If organisms lying on Cellophane are stained by means of dyes applied to the distal surface of the membrane, there is no obvious inequality in the depth of colour attained. Yet the dye molecules are much larger than any in the defined culture media.

(iv) From a generation time distribution it is possible to calculate the growth rate to be expected in a whole culture in the same medium and at the same temperature. In the few cases where comparison with a directly and accurately determined growth rate has been made, the two values have agreed within about 2 %, and that determined from the generation time distribution has been the greater (but not significantly so). The average permeability of Cellophane is thus high enough not to restrict the access of nutrilites to the inoculum; if there are patches of effectively low permeability, their total area must be small.

(v) Most enzyme reactions, and in fact the reactions of intact organisms with their nutrilites, obey a law of the Michaelis type. If S is the concentration of a substrate or nutrilite, t the time, and k and K are constants,

$$-\frac{dS}{dt}=k\frac{S}{K+S}. \tag{1}$$

Usually K is exceedingly small, and dS/dt is nearly constant for $S \gg K$ (compare, for example, Dagley & Hinshelwood, 1938), so moderate inequalities in the permeability of the membrane should not affect the growth rate, unless the passage of nutrilites is everywhere much impeded.

Constancy of growth rate

I have previously pointed out that the distribution of generation times can be usefully investigated only when the growth rate is constant. In each series of experiments I therefore carried out a rather crude test for constancy of growth rate: most experiments were continued for a time sufficient to include two successive generations of organisms; evidently the mean for the first generation (the mothers) should be the same as the mean for the second generation (the daughters). Taking all the experiments of one series together, suppose there are n_m mothers, of mean $\bar{\tau}_m$, and n_d daughters, of mean $\bar{\tau}_d$. Under the null hypothesis that both generations have the same distribution of τ, we can take for var τ the value obtained from the whole sample, and carry out a t-test for the significance of $|\bar{\tau}_m-\bar{\tau}_d|$. Normally we should have

$$\operatorname{var}(\bar{\tau}_m-\bar{\tau}_d)=(1/n_m+1/n_d)\operatorname{var}\tau,$$

but here the real variance of the difference is much greater because of correlation between generation times of related organisms. The principal factor which increases the variance is the strong positive correlation between sisters (Table 4B). Now almost without exception both mothers and daughters occur as S-dyads in the sample (cf. Fig. 2), and if τ_{11}, τ_{12}, are the generation times of a dyad,

$$\begin{aligned}\operatorname{var}(\tau_{11}+\tau_{12})&=2\{\operatorname{var}\tau+\operatorname{cov}(\tau_{11},\tau_{12})\}\\&=2\operatorname{var}\tau\{1+\rho(S)\},\end{aligned}$$

where $\rho(S)$ is the coefficient of correlation between sisters. Thus the variance of the mean for the pair is greater than $(\operatorname{var}\tau)/2$ by the factor $1+\rho(S)$. The variance of the mean for a sample of n, consisting of S-dyads only, and in the absence of other appreciable correlations, is not $(\operatorname{var}\tau)/n$ but $\{1+\rho(S)\}(\operatorname{var}\tau)/n$. Hence we have more nearly

$$\operatorname{var}(\bar{\tau}_m-\bar{\tau}_d)=(1/n_m+1/n_d)\{1+\rho(S)\}\operatorname{var}\tau.$$

Example 1. *Series Ba* 1

$\bar{\tau}_m=30{\cdot}39$; $n_m=123$.
$\bar{\tau}_d=29{\cdot}41$; $n_d=246$.
$\bar{\tau}_m-\bar{\tau}_d=0{\cdot}98$; var $\tau=36{\cdot}92$ (Table 10).
Estimate of $\rho(S)=0{\cdot}58$ (Table 4).
$(1/n_m+1/n_d)\operatorname{var}\tau=0{\cdot}4502$,
$\operatorname{var}(\bar{\tau}_m-\bar{\tau}_d)=0{\cdot}7098$,
Student's $t=1{\cdot}18$; $P(t)=0{\cdot}24$.

In four of the sixteen series thus tested, the t-probability was below the 5 % level, but of these four, three exhibited appreciable negative mother-daughter correlation (Series Ba 2, Cp 3, Pp 1, Table 4C). Such correlation tends to exaggerate the difference between the means. As the sign of $\bar{\tau}_m - \bar{\tau}_d$ was positive in eight series, negative in the other eight, and as a correction for the very appreciable correlation between cousins (Table 4D) would have reduced the t-values still further, I have assumed throughout that the growth rates have been constant.

ANALYSIS OF THE DATA

The example of the previous section shows how very much the failure of independence within the sample can exaggerate the result of a significance test. The effect is tantamount to a decrease in the number of degrees of freedom. In the two sections next following I have applied significance tests in the conventional way without any allowance for internal correlation, and it will be seen that the probability levels reached are consequently often widely scattered, and include many very small values (internal correlation affects the apparent probability level most when the true value is already rather small). These tests are therefore useful for comparison only; the general inferences are based on uniformity of behaviour. The type of argument adopted may be expressed formally thus: on a null hypothesis H, the possible outcomes of a trial are P and N, say, each with probability $\frac{1}{2}$; n trials are made, and p_0 result in P. Then if p_0 differs sufficiently from $\frac{1}{2}n$, $\sim H$ is asserted. The probability of $p \leqslant p_0$ or $p \geqslant p_0$ for a given n can be read from tables of partial sums of the binomial series (e.g. National Bureau of Standards, 1949).

The treatment which I have been able to devise for the purposes of these sections is solely directed to the demonstration of results of the above kind. It is very desirable that some less clumsy kind of manipulation should be developed. The product-moment coefficient of correlation, in particular, seems inappropriate; but it has the virtues of familiarity and arithmetical simplicity.

Stability of sample means; heterogeneity

In my previous investigation, I submitted the data for each series to a two-way analysis of variance for 'within experiment' and 'between experiment' components, and found that the latter was significantly in excess, presumably because of lack of reproducibility of the growth-rate (or mean generation time). This appears to be true here also; see Example 2.

The variance σ_E^2 thus estimated I call the 'apparent inter-experiment variance'; it is a measure of the amount by which the mean generation time differs from one experiment to another. The sixteen values are listed in Table 2, A, but the F-ratios and their probabilities are not given because the latter are all below 0·001. The obvious conclusion is unduly pessimistic, however. To continue with the same data as in Example 2, a test for homogeneity was carried out by dividing the nine experiments into two groups, such that the number of observations in any experiment of the 'upper' group was greater than the number in any experiment of the 'lower' group. Two-way

Example 2. *Series Ba* 2

2-way analysis of variance

No. of experiments $= k = 9$.
No. of observations in jth expt. $= n_j = 21, 24, 31, 31, 36, 47, 48, 64, 70$.
Total no. of observations $= N = 372$.

Source of variance	Sum of squares	Degrees of freedom	Mean square	Variance estimated
Between experiments	1,169	8	146·1	$\sigma_\tau^2 + M\sigma_E^2$
Within experiments	8,968	363	24·71	σ_τ^2
Total	10,137	371	—	—

$$M = \frac{N^2 - \Sigma n_j^2}{N(k-1)} = 40{\cdot}538;\ F\text{-ratio} = 5{\cdot}91\ (8,\ 363);\ P(F) < 0{\cdot}001;\ \text{estimated } \sigma_E^2 = 2{\cdot}99.$$

Table 2. *Two-way analyses of variance*

A: estimated inter-experiment variance, all experiments together. B: F-ratio (and degrees of freedom), F-ratio probability, and estimated inter-experiment variance for large-sample experiments. C: the same for small-sample experiments. In A, the σ_E^2 are all significant at the 0·001 level. (See Examples 2 and 3.)

	A	B			C		
Series	Est. σ_E^2	$F\ (n_1, n_2)$	$P(F)$	Est. σ_{EU}^2	$F\ (n_1, n_2)$	$P(F)$	Est. σ_{EL}^2
Ba 1	6·27	5·95 (4, 209)	<0·001	4·18	13·65 (4, 153)	<0·001	10·5
2	2·99	3·32 (3, 225)	<0·025	1·08	8·82 (4, 138)	<0·001	6·07
3	11·4	5·92 (4, 206)	<0·001	7·56	5·21 (6, 100)	<0·001	21·6
4	11·9	6·52 (3, 246)	<0·001	9·79	4·84 (4, 139)	<0·001	20·5
5	2·10	3·08 (3, 198)	<0·001	0·926	8·03 (4, 116)	<0·001	4·96
6	8·71	11·03 (3, 223)	<0·001	7·07	17·02 (4, 146)	<0·001	11·7
CP 1	9·48	34·48 (3, 205)	<0·001	6·89	21·33 (4, 153)	<0·001	14·0
2	31·3	5·90 (3, 241)	<0·001	15·1	8·59 (4, 127)	<0·001	73·5
3	88·2	1·23 (3, 225)	>0·25	1·06	20·04 (4, 142)	<0·001	167
4	27·1	17·57 (3, 249)	<0·001	27·2	17·14 (4, 140)	<0·001	36·0
5	14·9	4·84 (3, 224)	<0·005	9·24	5·35 (3, 887)	<0·005	33·2
Sf 1	6·44	8·39 (3, 202)	<0·001	1·69	25·39 (4, 153)	<0·001	12·4
2	4·63	29·09 (4, 274)	<0·001	4·81	25·76 (5, 157)	<0·001	5·52
3	5·23	27·27 (3, 268)	<0·001	3·88	12·22 (4, 169)	<0·001	8·67
Pp 1	6·28	5·28 (4, 250)	<0·001	2·09	18·79 (5, 104)	<0·001	16·6
2	4·81	5·24 (3, 200)	<0·005	2·50	2·18 (5, 119)	>0·05	0·958

analysis of both groups separately gave the suggestive result that the inter-experiment variance was greater in the lower group (σ_{EL}^2) than in the upper (σ_{EU}^2) (see Example 3).

When this test was repeated with the other series, it was found that in only one case was σ_{EL}^2 less than σ_{EU}^2 (Table 2, B, C). If on the average σ_{EL}^2 were equal to σ_{EU}^2, the probability of so great a disparity would be 0·0005. It seems therefore that the mean of small samples is less stable than that of large, and that the apparent fluctuation is in part caused by some form of heterogeneity in the population. It may be inferred that if still larger samples could be handled in single experiments, the σ_E^2 would be still smaller. While it is too much to hope that the fluctuation resulting from lack of reproducibility is really zero, the inter-experiment variance is probably less than the σ_{EU}^2 of

Example 3. *Series Ba* 2

2-way analysis of upper group

$k=4$; $n_j=47, 48, 64, 70$; $N=229$.

Source of variance	Sum of squares	Degrees of freedom	Mean square	Variance estimated
Between experiments	263	3	87·67	$\sigma^2_{TU}+M\sigma^2_{EU}$
Within experiments	5933	225	26·37	σ^2_{TU}
Total	6196	228	—	—

$M=56\cdot67$; F-ratio $=3\cdot32$ (3, 225); $P(F)<0\cdot001$; estimated $\sigma^2_{EU}=1\cdot08$.

2-way analysis of lower group

$k=5$; $n_j=21, 24, 31, 31, 36$; $N=143$.

Source of variance	Sum of squares	Degrees of freedom	Mean square	Variance estimated
Between experiments	776	4	194·00	$\sigma^2_{TL}+M\sigma^2_{EL}$
Within experiments	3035	138	21·99	σ^2_{TL}
Total	3811	142	—	—

$M=28\cdot35$; F-ratio $=8\cdot82$ (4, 138); $P(F)<0\cdot001$; estimated $\sigma^2_{EL}=6\cdot07$.

Table 2, B, and in most cases is probably a small fraction of the population variance (compare the σ^2_{EU} of Table 2, B with the estimated μ_2 of Table 10). In what follows, therefore, I assume that the true inter-experiment variance lies between σ^2_E and 0. So far this assumption is only an extrapolation. However, the dependence of apparent inter-experiment variance on sample size can be plausibly explained as a result of positive association between the generation times of nearly related organisms (and more specific reasons for adopting this explanation will appear in the next section). The selection of organisms for observation is not a random process; reference to the section on method will show that it must frequently happen that all the organisms selected at the beginning of an experiment come from one clone, the progeny of a single cell of the inoculum; and this is more likely to be true, the smaller the number of organisms chosen—a large number is more likely to derive from several clones. The explanation is in accord with the impression given by the earlier work, viz. 'a very large family tree, representing the numerical and temporal extension of a culture, consists of a patchwork of areas within some of which growth is steady, while within others it is erratic' (Powell, 1955); it can be tested by an extension of the analysis of variance. If we call a group of closely related organisms a 'family', we can determine by a three-way analysis the significant existence of inter-family as well as inter-experiment variances. It is obviously convenient to choose as the family units the F-hexads (Fig. 2*a*) into which most of the observations were naturally grouped (see Example 4).

In eleven of the sixteen series, the F_F-ratio probability was below 0·05, and in only two was the ratio itself less than unity—fairly strong evidence of a real inter-family variance (Table 3, A). In this analysis we have, by admitting a variance σ^2_{E3}, proceeded on the assumption that appreciable inter-experiment

Example 4. *Series Cp* 4

3-way analysis of variance

No. of experiments $= k = 9$.
No. of F-hexads in jth expt. $= n_j = 12, 12, 8, 5, 5, 4, 4, 4, 2$.
Total no. of observations $= N = 6\Sigma n_j = 336$.

Source of variance	Sum of squares	Degrees of freedom	Mean square	Variance estimated
Between experiments	7,749	8	968·6	$\sigma^2_{\tau 3} + 6\sigma^2_{F3} + M\sigma^2_{E3}$
Within experiments and between hexads	9,226	47	196·3	$\sigma_{\tau 3} + 6\sigma^2_{F3}$
Within hexads	17,850	280	63·75	$\sigma^2_{\tau 3}$
Total	34,825	335	—	—

$$M = 6\,\frac{(\Sigma n_j)^2 - \Sigma n_j^2}{(\Sigma n_j)(k-1)} = 35{\cdot}920.$$

$$\frac{\sigma^2_{\tau 3} + 6\sigma^2_{F3}}{\sigma^2_{\tau 3}} = F_F = 3{\cdot}08\ (47,\ 280);\ P(F_F) < 0{\cdot}001.$$

$$\frac{\sigma^2_{\tau 3} + 6\sigma^2_{F3} + M\sigma^2_{E3}}{\sigma^2_{\tau 3} + 6\sigma^2_{F3}} = F_E = 4{\cdot}93\ (8,\ 47);\ P(F_E) < 0{\cdot}001.$$

Estimated $\sigma^2_{F3} = 22{\cdot}09$.
Estimated $\sigma^2_{E3} = 21{\cdot}50$.

variance exists. But there is no reason to suppose that the F-hexad has any special significance, and if, as is likely, the association extends beyond the limits of this group, part of the σ^2_{E3} will be contributed by inter-family variance. Assuming for the time being that the real inter-experiment variance is zero, we can combine the first two lines of the 3-way analysis of Example 4.

Example 5. *Series Cp* 4

2-way analysis of variance

No. of F-hexads $= 56$.
No. of observations $= 336$.

Source of variance	Sum of squares	Degrees of freedom	Mean square	Variance estimated
Between hexads	16,975	55	308·63	$\sigma^2_{\tau 2} + 6\sigma^2_{F2}$
Within hexads	17,850	280	63·75	$\sigma^2_{\tau 2}$
Total	34,825	335	—	—

F-ratio $= 4{\cdot}84\ (55,\ 280)$; $P(F) < 0{\cdot}001$; estimated $\sigma^2_{F2} = 40{\cdot}81$.

The estimates of σ^2_{F2} so found are given in Table 3, B. We may tentatively regard σ^2_{F2} and σ^2_{F3} as upper and lower limits for a quantity, the 'inter-family variance', which measures in a gross but comprehensive way the extent to which generation times are associated as a result of hereditary effects. (I do not imply that the inheritance is necessarily nuclear or chromosomal in character.)

Inspection of the records suggests also that properly conducted tests for homogeneity of variance would disclose similar regularities in the relation between sample size and the Bartlett criterion. Unfortunately the significance of the criterion can be very much altered by a correction for the kurtosis of the

Table 3. *Two- and three-way analyses of variance*

A: for between experiment, between family and within family components. The ratio F_F determines the significance of the inter-family variance σ^2_{F3}, F_E the significance of the inter-experiment component σ^2_{E3}. B: for between and within family components. (See Examples 4 and 5.)

Series	No. of expts.	No. of *F*-hexads	σ^2_T (common to A and B)	A						B		
				σ^2_{F3}	σ^2_{E3}	F_F	$P(F_F)$	F_E	$P(F_E)$	σ^2_{F2}	F	$P(F)$
Ba 1	10	60	28·7	3·31	6·28	1·69	0·001	5·56	< 0·001	8·94	2·87	< 0·001
2	9	62	24·0	(0)	2·95	0·85	—	6·75	< 0·001	1·93	5·84	0·01
3	7	30	50·4	8·51	19·5	2·01	< 0·05	4·24	0·005	19·8	3·36	< 0·001
4	8	65	94·6	1·66	17·5	1·11	> 0·25	8·98	< 0·001	16·9	2·07	< 0·001
5	9	47	15·70	2·10	3·00	1·80	< 0·01	4·23	0·001	4·75	2·82	< 0·001
6	9	60	25·0	6·79	6·54	2·63	< 0·001	4·91	< 0·001	12·6	4·03	< 0·001
Cp 1	9	61	13·8	3·61	9·73	2·57	< 0·001	11·99	< 0·001	12·3	6·33	< 0·001
2	8	47	130·1	1·30	49·1	1·06	> 0·25	13·0	< 0·001	43·3	3·00	< 0·001
3	4	22	228	10·7	(0)	1·28	> 0·1	0·86	—	9·78	1·26	> 0·1
4	9	56	63·8	22·1	21·5	3·08	< 0·001	4·93	< 0·001	40·8	4·84	< 0·001
5	7	34	112	30·4	9·81	2·62	< 0·001	1·93	> 0·1	38·7	3·07	< 0·001
Sf 1	9	56	12·2	2·11	6·46	2·04	0·001	10·58	< 0·001	7·87	4·88	< 0·001
2	10	70	6·63	1·99	4·36	2·80	< 0·001	10·72	< 0·001	5·92	6·36	< 0·001
3	9	70	11·1	2·22	4·06	2·20	< 0·001	8·59	< 0·001	5·80	4·14	< 0·001
Pp 1	8	45	18·8	(0)	4·71	0·86	—	10·7	< 0·001	3·68	2·15	< 0·001
2	8	37	21·8	8·96	3·08	3·46	< 0·001	2·04	> 0·05	11·5	4·17	< 0·001

presumptive population (see, for example, Box, 1953). Here the kurtosis is so poorly determined as to be quite unreliable; the sample values of γ_2 (Table 10) range from $-0{\cdot}2$ to $8{\cdot}1$, and show no obvious association with species or experimental conditions. For the purpose of estimating fourth moments the sample sizes are already small, and are effectively reduced further by internal correlation. Hence in the absence of any acceptable hypothesis about the population distribution, no conclusions can be drawn from the Bartlett test.

Correlation between generation times

Four correlations having a simple significance can be computed from the data available, viz. the correlations between mothers and daughters ($\rho(H)$), between sisters ($\rho(S)$), between first cousins ($\rho(C_1)$) and between second cousins ($\rho(C_2)$). But before the results can be presented, certain difficulties require discussion.

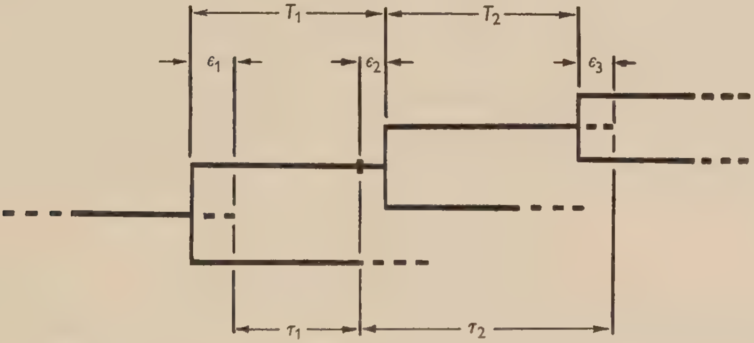

Fig. 3. Effect of errors, ϵ, on correlation between generation times. τ_1, τ_2: measured values; T_1 T_2; true values.

In Fig. 3, T_1 and T_2 represent the true generation times of a mother and a daughter cell respectively. If the corresponding estimated times of fission are in error by ϵ_1, ϵ_2, ϵ_3, the recorded generation times will be τ_1, τ_2, where

$$\tau_1 = T_1 + \epsilon_2 - \epsilon_1,$$

$$\tau_2 = T_2 + \epsilon_3 - \epsilon_2.$$

Now if we assume as we reasonably may, that the ϵ are all distributed alike and are independent of the T and of one another,

$$\operatorname{var} \tau = \operatorname{var} T + 2 \operatorname{var} \epsilon,$$

$$\operatorname{var} (\tau_1 + \tau_2) = 2 \operatorname{var} T + 2 \operatorname{cov} (T_1, T_2) + 2 \operatorname{var} \epsilon,$$

and the observed mother-daughter correlation will be

$$\rho(H) = \frac{\operatorname{var} (\tau_1 + \tau_2)}{2 \operatorname{var} \tau} - 1$$

$$= \frac{\operatorname{cov} (T_1, T_2) - \operatorname{var} \epsilon}{\operatorname{var} T + 2 \operatorname{var} \epsilon}. \qquad (2)$$

Thus $\rho(H)$ will be biased towards the value $-\frac{1}{2}$ by the common error (ϵ_2) which affects τ_1 and τ_2 in opposite ways. Analogous reasoning shows that the correlation between sisters is biased towards $+\frac{1}{2}$ by the common error in determining the time of inception:

$$\rho(S) \text{ (observed)} = \frac{\operatorname{cov}(T_1, T_2) + \operatorname{var} \epsilon}{\operatorname{var} T + 2 \operatorname{var} \epsilon}, \tag{3}$$

where now T_1, T_2 refer to the true generation times of an S-dyad. Because of the uncertain magnitude of var ϵ, I have computed estimates of $\rho(H)$ and $\rho(S)$ from the crude moments of the paired observations, but it is highly improbable that 2 var ϵ much exceeds the usual Sheppard correction for grouping (which it includes) or that its neglect had led to wrong conclusions. However, this point will need closer attention when it becomes possible to treat $\rho(H)$ and $\rho(S)$ in quantitative detail.

The correlation coefficients are also subject to a bias (always towards positive values) as a result of inter-experiment variance. We may consider (cf. Powell, 1955) the generation times measured in any one experiment to be a sample from a distribution $h(\tau, a, c_0)$ whose mean a varies from experiment to experiment but whose coefficient of variation c_0 is constant. (In the next section it will be seen that there are good grounds for this assumption.) Let $j(a)$ be the frequency function of the mean, c_j its coefficient of variation, and let $\boldsymbol{a}$ and c be the grand mean and coefficient of variation of all the observations. Then

$$c^2 + 1 = (c_0^2 + 1)(c_j^2 + 1), \tag{4}$$

and

$$\boldsymbol{a} = \iint \tau h(\tau, a, c_0) j(a)\, da\, d\tau = \int a j(a)\, da.$$

Now in a given experiment let

$$\mathsf{H}(\tau_1, \tau_2; a, a; c_0, c_0; \rho_0)$$

be the joint distribution of pairs of generation times τ_1, τ_2 (of a particular sort), and assume for the time being that the correlation ρ_0 is constant, or at least statistically independent of a. Write μ, $\mu(h)$, $\mu(\mathsf{H})$ for the moments of the overall distribution and of the h- and H-distributions. Then

$$\left.\begin{aligned} &\mu'_{10}(\mathsf{H}) = \mu'_{01}(\mathsf{H}) = \mu'_1(h) = a, \\ &\mu'_{20}(\mathsf{H}) = \mu'_{02}(\mathsf{H}) = \mu'_2(h) = a^2(c_0^2 + 1), \\ &\mu_{11}(\mathsf{H}) = \rho_0 \mu_2(h), \\ &\mu'_{11}(\mathsf{H}) = a^2(\rho_0 c_0^2 + 1). \end{aligned}\right\} \tag{5}$$

The value of the correlation coefficient calculated from all the observations will be an estimate of

$$\frac{\mu_{11}}{\mu_2} = \rho, \text{ say.}$$

But

$$\begin{aligned} \mu'_{11} &= \int a^2(\rho_0 c_0^2 + 1) j(a)\, da \quad \text{(from (5))} \\ &= \boldsymbol{a}^2(\rho_0 c_0^2 + 1)(c_j^2 + 1); \\ \mu_{11} &= \boldsymbol{a}^2(\rho_0 c_0^2 c_j^2 + \rho_0 c_0^2 + c_j^2), \end{aligned}$$

while

$$\mu_2 = \boldsymbol{a}^2 c^2.$$

Hence, using (4)
$$\rho = \rho_0 + (1-\rho_0)\, c_j^2/c^2, \tag{6}$$
or
$$\rho_0 = \rho - (1-\rho)\, c_j^2/(c^2 - c_j^2). \tag{7}$$
(It may be noted that $c_j^2/c^2 = \text{var } a/\text{var } \tau$, where var a is the inter-experiment variance.)

There is no very good reason to suppose that ρ_0 really is independent of a, though the constancy of c_0 suggests it. However, we may safely deny a null hypothesis that $\rho_0 \equiv 0$ if ρ differs sufficiently from c_j^2/c^2. If, on the other hand, apparently $\rho = c_j^2/c^2$ it does not follow that $\rho_0 \equiv 0$, since the observed equality could be produced by a ρ_0 varying appropriately with a.

There is some difficulty in the choice of a coefficient to represent the correlation between cousins and between second cousins. Two measures suggest themselves immediately: (i) we may compute correlations $\rho(C_1)$, $\rho(C_2)$, from all possible pairs; a C_1-tetrad provides four pairs, a C_2-octad sixteen (cf. Fig. 2), but not all are independent; (ii) we may compute correlations $\rho(\overline{C}_1)$, $\rho(\overline{C}_2)$ between the means of the members on either side of the family, so that each tetrad or octad yields only one pair. The advantage of $\rho(C_1)$, $\rho(C_2)$ is that the correction for inter-experiment variance (Equations 5 and 6) applies to them directly. Moreover, pairs from incomplete tetrads and octads may be included in the sample. But, like the usual intraclass correlation coefficient, they are restricted in range; in fact
$$|\rho(C_1)| \leqslant \tfrac{1}{2}\{1+\rho(S)\};$$
$$|\rho(C_2)| \leqslant \tfrac{1}{4}\{1+\rho(S)\}\{1+\rho(\overline{C}_1)\}.$$
The correlations $\rho(\overline{C}_1)$, $\rho(\overline{C}_2)$ between means have the full possible range -1 to $+1$. Corresponding to Equation (6) we have for the effect of inter-experiment variance
$$\rho(\overline{C}_1) = \rho_0(\overline{C}_1) + \{1-\rho_0(\overline{C}_1)\}\frac{2c_j^2}{c^2\{1+\rho(S)\}},$$
$$\rho(\overline{C}_2) = \rho_0(\overline{C}_2) + \{1-\rho_0(\overline{C}_2)\}\frac{4c_j^2}{c^2\{1+\rho(S)\}\{1+\rho(\overline{C}_1)\}}.$$
So far as the whole population is concerned, these formulae are a mere algebraic transformation of Equation (6), (with $\rho = \rho(C_1)$ etc.), but when the ρ are replaced by sample values, they become very sensitive to sampling fluctuation because of the extra factors in the last terms on the right. Consequently I have preferred the coefficients $\rho(C_1)$, $\rho(C_2)$, formed from all possible pairs of cousins and second cousins. These coefficients, because of their limited range, reflect only weakly the similarity between the two branches of a family stemming from one ancestor.

In order not to give a wrong impression of the sample sizes, I have adopted a special notation in Table 4, C, D, E. Thus a mother-daughter correlation is mostly derived from H-triads (τ_{11}, τ_{211}, τ_{212}; Fig. 2a), each yielding two pairs with a common member, but in any series there are some isolated pairs; the sample size is reported as $A_3 + B_1$, where A is the number of H-triads and B the number of isolated pairs; the total number of pairs is $2A+B$. For first cousins, $A_4 + B_1$ means that the sample consists of A complete C_1-tetrads plus a number of variously incomplete tetrads providing B extra pairs; total $4A+B$. Similarly, for second cousins.

The distribution of the τ is always markedly skew, and it might at first seem better to compute correlation coefficients from $\log \tau$, which is a nearly normal variate. But for the present merely synoptic purpose the untransformed variate has the advantage that the correlations computed from it are affected in a simple way by the different sources of error.

Table 4. *Estimates of the product-moment correlation coefficients between mothers and daughters* ($\rho(H)$), *sisters* ($\rho(S)$), *first cousins* ($\rho(C_1)$) *and second cousins* ($\rho(C_2)$)

The factor c_j^2/c^2 is calculated from the σ_E^2 of Table 2A and the μ_2 of Table 10.

	A	B		C		D		E	
Series	c_j^2/c^2	$\rho(S)$ +	Sample size	$\rho(H)$	Sample size	$\rho(C_2)$ +	Sample size	$\rho(C_2)$	Sample size
Ba 1	0·170	0·576	185	−0·036	122_3+2_1	0·411	73_4+4_1	+0·309	16_8+6_1
2	0·111	0·603	184	−0·316	127_3+1_1	0·207	71_4+2_1	+0·099	16_8
3	0·151	0·355	155	+0·166	65_3+5_1	0·256	47_4+5_1	+0·087	13_8+20_1
4	0·0896	0·606	195	−0·093	131_3+1_1	0·306	89_4+1_1	+0·357	30_8+4_1
5	0·0970	0·609	161	+0·015	97_3	0·364	72_4	+0·164	36_8+8_1
6	0·222	0·419	186	+0·312	125_3+6_1	0·292	85_4+9_1	+0·120	23_8+50_1
Cp 1	0·406	0·698	182	+0·302	125_3+1_1	0·556	71_4+4_1	+0·199	13_8+8_1
2	0·147	0·570	187	+0·014	96_3+3_1	0·436	77_4+3_1	−0·122	17_8+16_1
3	0·267	0·735	185	−0·186	48_3+2_1	0·496	80_4+7_1	+0·318	15_8+32_1
4	0·246	0·791	196	+0·200	116_3+5_1	0·680	79_4+8_1	+0·391	23_8+36_1
5	0·0942	0·592	159	+0·085	69_3+1_1	0·478	75_4+2_1	+0·224	19_8
Sf 1	0·343	0·769	180	+0·152	116_3+3_1	0·610	80_4+8_1	+0·508	21_8+21_1
2	0·386	0·804	220	+0·182	142_3	0·629	106_4+2_1	+0·335	35_8+8_1
3	0·255	0·758	222	+0·146	142_3+1_1	0·493	98_4+4_1	+0·292	28_8+12_1
Pp 1	0·230	0·582	181	−0·120	95_3+3_1	0·266	74_4+4_1	+0·283	23_8+12_1
2	0·167	0·780	161	+0·016	78_3+5_1	0·418	71_4+10_1	+0·359	23_8+57_1

The crude estimates of the four correlations are given in Table 4, B, C, D, E, together with (A) the factor $c_j^2/c^2 = \text{var } a/\text{var } \tau$. Here var a is taken as the estimated σ_E^2 of Table 2, A, which is almost certainly considerably greater than the true inter-experiment variance, and var τ is the estimated μ_2 of the whole distribution (Table 10). All the $\rho(S)$ exceed c_j^2/c^2, often by a large margin; of the $\rho(H)$ all but two are less than c_j^2/c^2, but the differences are on the whole smaller. All the $\rho(C_1)$ also exceed c_j^2/c^2 by a moderate amount. The corrected estimates of $\rho_0(S)$, $\rho_0(H)$ in Table 5, A were obtained by application of Equation (7). We see that the correlation between sisters is certainly strongly positive and between cousins it is appreciably so. Between mothers and daughters it is more variable but usually near zero—the uncertainty about the true value of σ_E^2 (and so of c_j^2/c^2) makes it impossible to say whether usually positive or negative. The combination of positive $\rho(S)$ and $\rho(C_1)$, perhaps offset by a small negative $\rho(H)$, itself suggests that the group of six generation times forming a F-hexad might exhibit a smaller variance than a group of six chosen at random. The conclusion of the previous section, that an inter-family variance exists (or, equally, that the τ of closely related organisms are associated), is corroborated.

Of the estimates of $\rho(C_2)$ (Table 4, E) all but one are positive, but only ten exceed c_j^2/c^2, not in itself an unlikely event. Considering that the adopted value of c_j^2/c^2 is probably too large we may perhaps admit a weak implication that second cousins are positively associated.

Table 5. *Estimates of correlations corrected for inter-experiment variance* $(\rho_0(S), \rho_0(H), \rho_0(C_1))$ *and for inter-family variance* $(\rho_F(S), \rho_F(H))$

The factor c_k^2/c^2 is calculated from the σ_{F2}^2 of Table 3, B and the μ_2 of Table 10.

	A			B	C	
Series	$\rho_0(S)$ +	$\rho_0(H)$	$\rho_0(C_1)$ +	c_k^2/c^2	$\rho_F(S)$ +	$\rho_F(H)$ −
Ba 1	0·490	−0·248	0·291	0·242	0·441	0·367
2	0·553	−0·481	0·108	0·072	0·572	0·418
3	0·240	+0·018	0·124	0·263	0·125	0·132
4	0·567	−0·201	0·238	0·127	0·549	0·252
5	0·567	−0·091	0·296	0·219	0·500	0·261
6	0·253	+0·115	0·090	0·322	0·144	0·014
Cp 1	0·491	−0·173	0·252	0·526	0·362	0·472
2	0·496	−0·156	0·339	0·203	0·460	0·237
3	0·638	−0·618	0·313	0·030	0·727	0·222
4	0·722	−0·061	0·576	0·370	0·668	0·270
5	0·550	−0·010	0·424	0·245	0·460	0·212
Sf 1	0·648	−0·290	0·406	0·419	0·603	0·459
2	0·680	−0·333	0·395	0·494	0·612	0·617
3	0·676	−0·147	0·319	0·283	0·663	0·192
Pp 1	0·456	−0·383	0·047	0·135	0·516	0·257
2	0·736	−0·182	0·301	0·400	0·633	0·639

Thus a given organism has a part in determining the particular generation times of its progeny, certainly down to the second, and perhaps to the third generation.

We may now think of the mean τ of a F-hexad as an approximate measure of the local growth rate, and the σ_{F2}^2, σ_{F3}^2 of Table 3 as measures of its fluctuation; it becomes of interest to ask further, what is the strength of the *local* association (i.e. relative to the local $\bar{\tau}$) between sisters and between mothers and daughters? The required estimates may be obtained by applying Equation (7) again to $\rho(S)$ and $\rho(H)$, but now the coefficient of variation of the experiment means, c_j^2, must be replaced by the coefficient of variation of F-hexad means, say c_k^2, obtained from the σ_{F2}^2 of Table 3, B. The two correlations $\rho_F(S)$, $\rho_F(H)$ so corrected for inter-family variance are given in Table 5, C; the $\rho_F(H)$ are uniformly negative and generally comparable in magnitude with, though less than, the $\rho_F(S)$. This is most unusual. If an organism possesses a regulating mechanism which influences a property of its immediate progeny their similarity to itself is not necessarily secured thereby, but similarity is very generally observed, and this is as true of manners or money as of genes. (Egregious exceptions such as are found in sporozoa and cryptogamia are explicable in genetic terms.) It may be objected that the notion of a local growth rate and its attendant variances is factitious, but there is *prima facie* evidence for its propriety. Comparison of $\rho(H)$ (Table 4, C) with c_k^2/c^2 (Table 5, B) suggests that the (algebraically) smaller $\rho(H)$ are associated with the smaller c_k^2/c^2; in fact the Spearman rank correlation between them is about $+0{\cdot}86$, significantly different from zero at the 0·001 level. Thus the less the $\rho(H)$ are affected by the inter-family variance (of the sample if not of the population) the smaller they appear to be. It seems that the difference in sign of the local correlations $\rho_F(S)$ and $\rho_F(H)$ is genuine, though their sample values are very poorly determined.

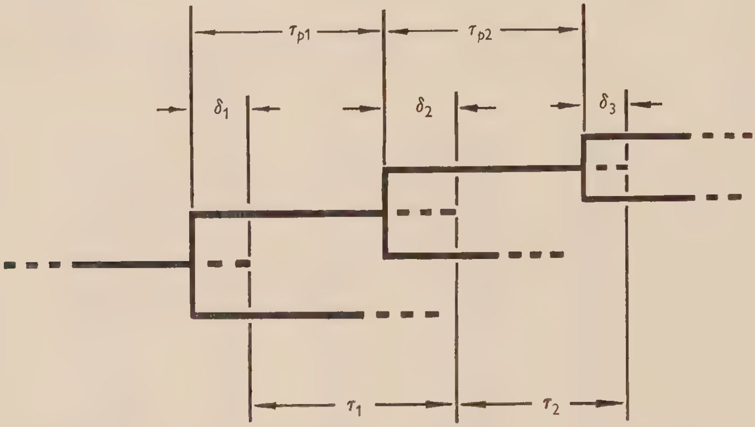

Fig. 4. Relation between observed *o*-generation times τ_1, τ_2 and *p*-generation times τ_{p1}, τ_{p2} as affected by 'delays' δ.

An explanation can be found by an examination of the process of fission (Fig. 1). It rests on the relation between the family tree whose nodes are *p*-fissions (say the *p*-tree) and the tree whose nodes are (observed) *o*-fissions (say the *o*-tree). In Fig. 4, the solid line represents part of a *p*-tree; the intervals between fissions, τ_p, have the same significance as in Fig. 1. The *o*-fissions are separated by intervals δ from the corresponding *p*-fissions. (I assume for the time being that every δ is less than the τ_p with the same origin.) The τ, intervals between successive *o*-fissions, are the observed generation times. Then if τ_1, τ_2 refer to mother and daughter,

$$\begin{aligned}\operatorname{var}(\tau_1+\tau_2) &= \operatorname{var}\tau_{p1}+\operatorname{var}\tau_{p2}+\operatorname{var}\delta_1+\operatorname{var}\delta_3+2\operatorname{cov}(\tau_{p1},\tau_{p2})+2\operatorname{cov}(\tau_{p1},\delta_3)\\ &\quad+2\operatorname{cov}(\tau_{p2},\delta_3)-2\operatorname{cov}(\tau_{p1},\delta_1)-2\operatorname{cov}(\tau_{p2},\delta_1)-2\operatorname{cov}(\delta_1,\delta_3), \quad (8)\\ \operatorname{var}\tau &= \operatorname{var}\tau_p+2\operatorname{var}\delta+2\operatorname{cov}(\tau_{p1},\delta_2)-2\operatorname{cov}(\tau_{p1},\delta_1)-2\operatorname{cov}(\delta_1,\delta_2).\end{aligned}$$

If now the covariances between the δ, and between them and the τ_p are neglected, and the variances are referred to the local mean τ,

$$\rho_F(H)=\frac{\operatorname{cov}(\tau_{p1},\tau_{p2})-\operatorname{var}\delta}{\operatorname{var}\tau_p+2\operatorname{var}\delta}, \qquad (9)$$

and similarly (precisely as in Equations (2) and (3)) for sisters

$$\rho_F(S) = \frac{\operatorname{cov}(\tau_{p1}, \tau_{p2}) + \operatorname{var}\delta}{\operatorname{var}\tau_p + 2\operatorname{var}\delta}. \tag{10}$$

Because of the symmetry of the joint distribution, $\operatorname{cov}(\tau_{p1}, \tau_{p2}) \leqslant \operatorname{var}\tau_p$ and we have only to allow that $\operatorname{var}\tau_p < \operatorname{var}\delta$ to make $\rho_F(H)$ negative, even though we might expect the covariances in Equations (9) and (10) to be both positive and of similar magnitude.

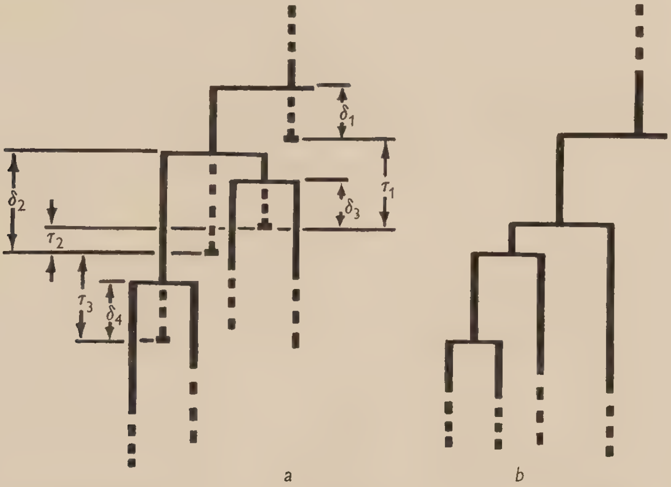

Fig. 5. Relation between the p-tree (a) (solid line) and the observed o-tree (b) when the δ are unrestricted.

I think that this explanation of the sign of $\rho_F(H)$ is correct in substance; although, regarded as a hypothesis, it is only feebly supported by the data, I can think of no phenomena with which it is in conflict. The formal analogy with the treatment of observational error (Equations (2) and (3)) is exact: var δ biases the observed ρ towards $+\frac{1}{2}$ or $-\frac{1}{2}$ as the case may be. We have, however, no knowledge of the covariances so summarily omitted, and we have again implicitly assumed that the ρ_F are independent of the local growth rate. The chief lesson of this discussion is that even if the τ_p are subject to a law of the Kendall or Rahn type, this fact will not necessarily manifest itself in relations between the τ. It is tempting to pursue the consequences of (9) and (10) further, but such speculation had better wait upon the outcome of more experimental work.

The condition that the δ shall be less than the corresponding τ_p is essential to the foregoing argument. Fig. 5a represents a p-tree with δ which are not so restricted. The p-fissions occur at the beginning of the δ intervals, the o-fissions at their end. The interval δ_2 is chosen long enough to overlap δ_3 and the preceding p-generation time, so that during δ_3 the tree represents an organism containing three protoplasts. (Three branches of the p-tree coexist with only one of the o-tree during δ_3.) The successive intervals τ_1, τ_2, τ_3 are recorded as

o-generation times, but τ_1 corresponds to no single o-generation time, and τ_2 should be regarded as negative. The observed o-tree is shown in Fig. 5*b*. Thus if there is no restriction on the δ the o- and p-trees are not in general homoeomorphic (in the topological sense) and though a unique one-one correspondence between their nodes must still exist, it cannot be inferred by inspection of the o-tree alone.

So large an aberration as is represented by δ_2 (Fig. 5*a*) is perhaps rare in unicellular organisms, but without special techniques it could only be directly recognized in cocci (Powell, 1955).

The status of the coefficient of variation

The coefficient of variation of generation time has a special significance in the hypotheses of Kendall and Rahn: it is closely related to the number of primitive steps in the postulated mechanism of fission. The relation is, for Kendall's hypothesis,

$$c^2 = 1/g, \tag{11}$$

and for Rahn's

$$c^2 = \frac{\Gamma_2(1) - \Gamma_2(1+g)}{\{\Gamma_1(1+g) - \Gamma_1(1)\}^2}, \tag{12}$$

where g is the number of primitive steps, c the coefficient of variation, and

$$\Gamma_n(x) = (d/dx)^n \log \Gamma(x).$$

Now that the measured generation times are recognized to pertain to secondary processes, the further examination of their coefficients of variation would seem unpromising; it turns out that a qualitative treatment exposes some features of interest.

Consider a single line of descent in a large family tree, and choose a time scale such that the mean $\bar{\tau}$ is unity. A segment of the line of temporal length n might represent about $n \pm \sqrt{(n \operatorname{var} \tau)}$ o-generation times, and $n \pm \sqrt{(n \operatorname{var} \tau_p)}$ p-generation times. But these numbers must not in any particular instance differ by more than two units if the morphology of the ancestor (supposed normal) is to be preserved; the o-fissions must keep nearly in step with the p-fissions, and the τ and τ_p must both regress (in Galton's sense) towards the same mean $\bar{\tau} = \bar{\tau}_p$. If τ_p and τ (or τ_p and δ) were independent variables, it would sometimes happen that a line of descent would contain more o-fissions than p-fissions; this is impossible, save for irregularity of one unit at the ends of the segment. Equally, the occurrence of more p-fissions than o-fissions would imply that multicellular individuals had been produced (Fig. 5), and the multicellular condition once attained might be inherited for an indefinite period (cf. Feller, 1950, p. 249 *seq.*). The observation that the morphology is preserved, with rare individual exceptions, shows that the variations in τ and τ_p are closely interlinked. Since in units of $\bar{\tau}$, var τ is c^2, we may expect a rough parallelism between $c^2(\tau)$ and $c^2(\tau_p)$ though this is of course not a logical necessity.

Incidentally, it is now evident that such covariances as cov (τ_{p1}, δ_1) in Equation (8) (see Fig. 4) are almost certainly not zero, though they may be negligible for the purpose of the argument there developed, which is restricted to local fluctuations.

The observed c^2 are listed in Table 7. They are all smaller than the c^2 derived from Kelly & Rahn (1932) or from my previous data, and imply, without any allowance for inter-experiment variance, that for Kendall's hypothesis (from Equation (11))

$$g=21 \text{ to } 57,$$

and for Rahn's (from Equation (12))

$$g=200 \text{ to } 8000.$$

Also given in Table 7 are coefficients of variation c_0 corrected for inter-experiment variance by means of Equation (4), c_j being taken as σ_E^2/a^2 (Table 2, A). The true coefficients probably lie between c and c_0. The c_0^2, which seem to be rather more uniform than the c^2, then lead to the following revised estimates of the g:

Kendall's $g=23$ to 93;

Rahn's $g=260$ to $130{,}000$.

These last figures demonstrate the insensitivity of c^2 (in Equation (12)) to changes in g, and the impracticability of determining the constancy of the latter. Still, Rahn's identification of his g with the number of genes in the organism now appears much more acceptable; such rough estimates of the number as can be made on other grounds (Haldane, 1954) fall within the above range. But the fact that the τ, and the high probability that the τ_p of related organisms, are associated deprives the data, in their purely numerical aspect, of all cogency.

For reasons already discussed in connexion with the homogeneity of variance, I have made no formal comparisons of the c^2, either between series or between experiments; instead, I have carried out two forms of test for stability, the first for independence of the local growth rate, the second for independence, under nominally fixed experimental conditions, of imperfect replication. The first test consisted in forming the correlation coefficient between the mean (say a_F) and the logarithm of the coefficient of variation (say $\log c_F$) of F-hexads, series by series. These two variates are approximately normally distributed. Table 6, A gives the estimated correlation coefficients together with the corresponding z ($=\tanh^{-1}\rho$) and their probabilities. The two extreme values lie outside the 0·05 probability level, but the weighted mean of the z is found to be $+0{\cdot}0023$—less than its standard error of 0·036—and seven of the sixteen are positive. The second test consisted in carrying out a two-way analysis of variance for 'between experiment' and 'within experiment' components of variance of $\log c_F$ (see Example 6). The results of this test (Table 6, B) show some excess of the higher F-ratios, but only one $P(F)$ is appreciably below 0·05, and six of the ratios are below unity.

Thus c_F is practically independent both of the local growth rate and of minor alterations in the cultural conditions. The coefficient of variation of the whole population will be greater than c_F to an extent depending on the

Table 6.

A: correlation coefficient between mean (a_F) and logarithm of coefficient of variation (c_F) of F-hexads, with z and its probability. B: F-ratios (and degrees of freedom) and probabilities for two-way analysis of variance of $\log c_F$. (See Example 6.)

	A				B	
Series	$\rho(a_F, \log c_F)$	No. of pairs	z	$P(z)$	$F, (n_1, n_2)$	$P(F)$
Ba 1	−0·053	60	−0·053	0·69	1·82 (9, 50)	>0·05
2	+0·004	62	+0·004	0·96	1·81 (8, 53)	*ca.* 0·1
3	−0·055	30	−0·055	0·77	1·40 (6, 23)	>0·25
4	−0·088	65	−0·088	0·49	0·44 (7, 57)	>0·1
5	−0·029	47	−0·029	0·85	2·21 (8, 38)	*ca.* 0·05
6	+0·270	60	+0·277	0·04	0·72 (8, 51)	>0·1
Cp 1	+0·398	60	+0·421	0·00	1·64 (8, 51)	>0·1
2	+0·031	47	+0·031	0·84	2·42 (7, 39)	*ca.* 0·05
3	+0·182	22	+0·184	0·42	2·47 (3, 18)	*ca.* 0·1
4	−0·197	56	−0·200	0·14	4·48 (8, 47)	<0·001
5	−0·234	34	−0·238	0·16	0·76 (6, 27)	>0·1
Sf 1	+0·151	56	+0·152	0·27	1·33 (8, 47)	>0·25
2	+0·091	68	+0·092	0·46	0·92 (9, 58)	>0·1
3	−0·066	70	−0·066	0·59	0·91 (8, 61)	>0·1
Pp 1	−0·375	45	−0·394	0·01	1·88 (7, 37)	*ca.* 0·1
2	−0·161	37	−0·162	0·32	0·25 (7, 29)	*ca.* 0·05

Example 6. *Series Sf* 3

2-way analysis of variance of $\log c_F$; c_F *the coefficient of variation of* τ *in F-hexads*

No. of experiments = 9.
Total no. of F-hexads = 70.
No. of F-hexads in individual experiments = 14, 12, 10, 8, 6, 6, 6, 4, 4.

Source of variance	Sum of squares	Degrees of freedom	Mean square
Between experiments	1·8274	8	0·2284
Within experiments	15·3886	61	0·2523
Total	17·2160	69	—

$F = 0{\cdot}9053$; $P(F) > 0{\cdot}1$.

inter-family variance and determined by an equation of the form (4); it can be constant only if the inter-family variance is so. We do not know if this is the case, but the two tests together suggest that the coefficient of variation may indeed reflect some special feature of the reproductive process.

The two series Ba 1 and Sf 1 which were carried out at a lower temperature than the rest (Table 1) are quite insufficient to show whether or not c (or c_0) varies with temperature; neither pair of values is extreme for the organism concerned (Table 7). Nor is there any clear indication that the chemical complexity of the medium affects c in a systematic way.

In Table 8, the coefficients of variation are classified according to organism and complexity of medium. The media TMB and PW are taken as 'complex' since they offer a variety of amino acids and atomic groupings which would

Table 7. *Coefficients of variation of the raw data* (c^2) *and corrected for apparent inter-experiment variance* (c_0^2)

Series	c^2	c_0^2
Ba 1	0·0422	0·0348
2	0·0451	0·0399
3	0·0312	0·0264
4	0·0485	0·0439
5	0·0287	0·0258
6	0·0344	0·0266
Cp 1	0·0446 –	0·0260
2	0·0319	0·0271
3	0·0446 +	0·0323
4	0·0384	0·0287
5	0·0276	0·0249
Sf 1	0·0206	0·0134
2	0·0176	0·0107
3	0·0277	0·0204
Pp 1	0·0282	0·0216
2	0·0198	0·0164

Table 8. *Coefficients of variation, uncorrected* (c^2) *and corrected* (c_0^2) *for apparent inter-experiment variance, classified according to organism and complexity of medium*

	$1000c^2$		$1000c_0^2$	
Organism	Complex media	Simple media	Complex media	Simple media
Aerobacter cloacae	42	29	35	26
	45	31	40	26
		34		27
		48*		44*
Serratia marcescens	45 –	28	26	25
		32		27
		38		29
		45 + *		32*
Pseudomonas aeruginosa	28	20	22	16

otherwise have to be synthesized by the organism; all the synthetic media are taken as 'simple'. Thirteen comparisons can be made between the c^2 for complex and the c^2 for simple media; in ten of these the complex medium gives the larger c^2. For c_0^2, the corresponding ratio is 8/13. The entries marked with an asterisk in Table 8 correspond to the medium HP (Series Ba 4 and Cp 3) containing sodium pyruvate; this medium appears to be somewhat toxic since it increases a and var τ, as well as c^2, relative to the simpler medium H (compare series Ba 3 and 4, Cp 2 and 3 in Table 10). If these entries are deleted, ten possible comparisons remain; in all of them c^2 is greater for the complex medium; for c_0^2, the ratio is 8/10. I do not think that much weight can be given to these latter comparisons; there is no satisfactory definition or criterion of toxicity, and certainly increased generation time alone is not necessarily indicative. There is, then, enough evidence to encourage a further study of the effect of medium on c^2, but no more. It is of the greatest importance that such a study should be undertaken. If it were found that the higher values of c^2 were systematically associated with the more complex growth media, the fact would constitute strong evidence in favour of a generalized hypothesis of the Kendall type, namely, that the dispersion of generation time is dependent on the number of synthetic processes the organism is called upon to perform in order to convert pabulum into living matter.

The incidence of non-viable organisms

There are two reasons for introducing this apparently alien subject:

(i) A healthy fully nourished culture produces only a small proportion of non-viable organisms, and it is therefore economical to record their occurrence on any occasion that offers. Very few satisfactory observations have ever been made; the classical methods for determining the proportion of non-viable organisms in a culture are susceptible to many kinds of error, and they are of little value when the proportion is small.

(ii) The fact that non-viable organisms are produced in favourable environments which are chemically and physically uniform gives them a title to inclusion in a description of the pattern of generation times. They can scarcely be ignored, for the effect of including them in the frequency function itself is to make the mean infinite.

It is not possible at present to set up a definition of viability. An adopted criterion must depend on reproductive power, since neither growth nor any chemical activity is uniquely a property of living organisms; furthermore, viability itself is not absolute, but only has a meaning in an environment which can be specified (Powell, 1956*b*). I submit the following remarks to provide a definite context for the few data I have to present, and so to avoid misunderstanding. The subject is fraught with a number of grave philosophical difficulties which have never been resolved and of which no mature discussion is yet possible. I believe that they can be resolved, if at all, only by an appeal to detailed observation.

I adopt the following terminology: a bacterium will be said to have been I-viable (from 'Individual') in such-and-such an environment if it is known to have undergone fission. This retrospective kind of statement is the only kind that can be made with certainty about the viability of an organism. The assertion of present viability is always doubtful, and of non-viability utterly precarious. (See, for example, Heinmets, Taylor & Lehman, 1954.) I assume, however, that an organism can meaningfully be said to be I-viable with probability α (or I-non-viable with probability $1-\alpha$) by induction from previous or contemporary experience. The state of an organism may be determinate, in which case α is merely a sampling frequency, or a chance element may enter the biological process itself; this is a matter for experiment to decide.

A knowledge of the viability of the individual in the above sense, i.e. of its capacity to undergo fission, is often of little interest to the biologist. More usually what is wanted is a knowledge of the probability that it will give rise to a large number of progeny after many generations. (Compare the discussion in Valentine & Bradfield, 1954.) I shall say that an organism has been S-viable (from 'Species') if it has given rise to an indefinitely long sequence of generations of progeny in such-and-such a constant environment. The uncertainty attached to the expression 'indefinitely long' could be diminished by a convention, but in many cases there is no practical objection to it. Suppose that in a culture growing under constant conditions a fraction α of the organisms which appear are I-viable, and that the occurrences of I-non-viable organisms are all independent of one another. Then if $\alpha \leqslant \frac{1}{2}$, the culture will die out. If $\alpha > \frac{1}{2}$, every I-viable organism is potentially S-viable, and it can properly be said that the species is viable in that environment. Feller (1950) proves a result equivalent to the following: when $1 > \alpha > \frac{1}{2}$ the number of nth generation progeny of an I-viable ancestor tends to infinity with n, or is 0, except in a vanishingly small proportion of cases. Under the assumptions of this example, the distinction between S-viability and S-non-viability is sharp: unless α differs by very little from $\frac{1}{2}$, an organism can be classified with a close approach to certainty after only a few generations of growth. Here also the relation between I-viability and S-viability is simple: the probability, β, that an I-viable organism will also be S-viable is given by

$$\left.\begin{aligned}\beta &= (2\alpha-1)/\alpha^2 \quad && (\alpha > \tfrac{1}{2}),\\ &= 0 && (\alpha \leqslant \tfrac{1}{2}).\end{aligned}\right\} \tag{13}$$

There are definite reasons why attention should first be fixed on the property of I-viability: (i) it appears to be more elementary than S-viability; (ii) it is easier, in working under the microscope, to establish constant and repeatable conditions for a short time than for a long time; (iii) given the laws of incidence of I-viability, those of S-viability can be inferred; the converse is in general false.

Henceforward I use 'viable' to mean I-viable. In the present context I call an organism non-viable (i) if it becomes only dimly visible under phase-contrast illumination and then shows no sign of growth during about 1 hr.; (ii) failing (i), if it shows no sign of growth during about 3 hr.

The experiments provide a sample of 3455 fissions, actually seen or known to have occurred in the culture chamber, and such that the fate of both organisms stemming from each is known. These are classified in Table 9. The index of viability, α, i.e. the proportion of viable organisms in the sample, is calculated for each species (α is one-half of the 'generation index', p, of *Topley & Wilson's Principles*, 1955). The number of non-viable organisms is too small to disclose any significant difference between species, still less between series; the grand total gives $\alpha = 0{\cdot}994$: among newly formed organisms probably less than 1 % are non-viable.

The index α is not the same as the proportion of viable organisms in a growing culture. In steady exponential growth the proportion tends, as the effect of the constitution of the inoculum is diluted out, to $2\alpha-1$ (see, for example, *Topley & Wilson's Principles*, 1955). The same value obtains in continuous cultures of the Monod (1950) type (Powell, 1956*c*). Here

$$2\alpha - 1 = 0{\cdot}988,$$

in good agreement with the value 0·984 obtained by Valentine & Bradfield (1954). Much lower values obtained by classical methods (e.g. 0·90; Wilson, 1922) have been widely accepted, but my own desultory experience with a few representatives of the genera *Bacillus*, *Mycobacterium*, *Corynebacterium*, *Staphylococcus*, *Salmonella* and *Pasteurella* suggests that α is very generally near 0·99 in unrestricted growth.

Table 9. *Incidence of non-viable organisms*

The index of viability α is the proportion of viable organisms in the sample.

		No. of fissions giving rise to		
Series	Total no. of fissions	1 non-viable organism	2 non-viable organisms	α
Ba 1	199	1	—	0·9950
2	194	2	—	
3	183	—	2	
4	222	2	—	
5	189	1	—	
6	213	2	—	
Cp 1	196	3	—	0·9945
2	217	—	—	
3	260	5	1	
4	220	1	—	
5	205	1	—	
Sf 1	212	4	2	0·9925
2	262	2	—	
3	255	1	—	
Pp 1	217	1	2	0·9918
2	211	2	—	
Totals	3455	28	7	0·9939

If we classify fissions as VV, NV and NN according as they produce none, one, or two non-viable organisms, we have for the expected proportions of these types

$$VV:NV:NN=\alpha^2:2\alpha(1-\alpha):(1-\alpha)^2,$$

or, in a sample of 3455 with $\alpha=0{\cdot}9939$,

$$VV:NV:NN=3413{\cdot}13:41{\cdot}74:0{\cdot}13.$$

The numbers observed are

$$VV:NV:NN=3420:28:7.$$

The expected number of type NN is too small to permit the use of a χ^2 test. Instead, we may reason thus: the expected number of type NN is so small that its sampling distribution will be approximately Poissonian. The standard error of $1-\alpha$ (42 of 6910 observations) is about 0·001—one-sixth of its value. Hence with great confidence we can say that the true expectation of NN is below 1, since $3455(1-\alpha)^2$ is only 0·13. But the chance of 7 or more in a Poisson distribution with parameter 1 is about 0·0006. Thus there is a strong indication that the viabilities of sisters are positively associated.

Of the 35 fissions giving rise to non-viable organisms (Table 9), 16 are the terminations of organisms of known generation time. Now the generation time of an organism one or both of whose progeny are non-viable will lie as often above as below the median of the τ distribution, if it is independent of the fate of the progeny. Allowing for possible inter-experiment variance, we should compare the τ of such organisms with the median τ of the experiment in which they occurred. It turns out that in 14 of the 16 available instances, the τ of the mothers lie above the experiment means and *a fortiori* (since the distributions are positively skew) above the medians. The probability of this occurrence is about 0·008 on the hypothesis of independence. It appears that a falling growth rate may precede the state of complete incompetence. The corresponding result for the viable sisters of non-viable organisms is neither striking nor significant (14 of 21 instances).

The most obvious way in which the incidence of non-viable organisms fails of being purely fortuitous is through the association between the fates of sisters. This phenomenon leads to a relation different from Equation (13) for the connexion between I-viability and S-viability. It is easy to show that, if β_n is the probability that an I-viable organism has I-viable progeny of the nth generation,

$$1-\beta_n=(1-\alpha)^2+2\alpha(1-\alpha)(1-\beta_{n-1})+\alpha^2(1-\beta_{n-1})^2, \tag{14}$$

where α is the index of viability, supposed constant, and where the occurrences of I-non-viability are supposed independent of one another. In the limit, as $n\to\infty$, (14) reduces to (13), giving the probability, β, that an I-viable organism is also S-viable. In (14) the coefficients $(1-\alpha)^2$, $2\alpha(1-\alpha)$, α^2 are the probabilities that both sisters of a pair are I-non-viable, that only one is so, and that neither is so, respectively. Hence we may write the limiting form of (14) more generally as

$$1-\beta=C+2B(1-\beta)+A(1-\beta)^2, \tag{15}$$

where now $AC\neq B^2$ if the fates of sisters are not independent. A convenient measure of association is Yule's (1912) coefficient of absolute association, V, which is closely related to χ^2 in a 2×2 contingency table:

$$V=\frac{AC-B^2}{(A+B)(B+C)}.$$

In (15)

$$A+2B+C=1,$$

$$A+B=\alpha,$$

and so

$$A=\alpha^2+\alpha(1-\alpha)V,$$

$$B=\alpha(1-\alpha)(1-V),$$

$$C=(1-\alpha)^2+\alpha(1-\alpha)V,$$

whence

$$\left.\begin{aligned}\beta&=\frac{2\alpha-1}{\alpha^2+\alpha(1-\alpha)V} & (\alpha>\tfrac{1}{2}),\\ &=0 & (\alpha\leqslant\tfrac{1}{2}).\end{aligned}\right\} \tag{16}$$

So the effect of positive association between sisters is to reduce the probability that an I-viable organism is also S-viable. For the totals of Table 9, V is 0·329, and from (16), with $\alpha = 0{\cdot}9939$, $\beta = 0{\cdot}998$.

When $V = 0$ (Equation 13)

$$\beta = 1{\cdot}000 - .$$

Only if α is less than about 0·9 will β be practically distinguishable from unity, whatever V is.

These considerations may be of some importance in the conventional method of determining a viable count by 'plating-out'. The method determines the number of organisms in the sample which are S-viable under the conditions of the test. Of course equations such as (13) or (16) are not likely to be obeyed exactly, because of the erratic growth of organisms on transfer to a new environment, and because in ordinary practice the supply of nutrients is not held constant; but the distinction between S-viability and I-viability and the probable change in α and β in the new environment ought to be remembered whenever it is known or suspected that $\alpha < 0{\cdot}9$.

The distribution of generation times

In the absence of a satisfactory working hypothesis, and in view of the uncertainty about inter-experiment variance, the data may as well be represented by any frequency function which fits adequately and which is practically convenient. In the choice of suitable functions I have been guided by the increasing importance of 'continuous culture' as a method of studying growth and of conducting large-scale fermentations. A knowledge of the generation time distribution is of great value in understanding the behaviour of such cultures (Powell, 1956*c*). Through this section I use $f(\tau)$ as a general notation for a frequency function of generation times, exclusive of non-viable organisms. Another, derived, generation time distribution, $\mathscr{C}(\tau)$ which I have called the 'carrier distribution' is important in determining the selective properties of continuous cultures. In the absence of appreciable mother-daughter correlation, and neglecting the small proportion of non-viable organisms,

$$\mathscr{C}(\tau) \equiv 2e^{-\nu\tau} f(\tau), \tag{17}$$

where ν is the number growth-rate constant, defined by

$$\int_0^\infty e^{-\nu\tau} f(\tau)\, d\tau = \tfrac{1}{2}.$$

Equation (17) limits the forms of $f(\tau)$ which can be conveniently handled to those having simple characteristic functions (or Laplace transforms); it excludes the Pearson Type VI and the otherwise attractive 'lognormal' distributions. Of the familiar distributions which are also singly terminated there remain the Pearson types III:

$$f(\tau) \equiv \frac{\tau^{g-1} e^{-\tau/m}}{m^g \Gamma(g)}, \tag{18}$$

and V:

$$f(\tau) \equiv \frac{m^g \tau^{-g-1} e^{-m/\tau}}{\Gamma(g)}. \tag{19}$$

A possible alternative to the Pearson Type VI is the three-parameter distribution

$$f(\tau) \equiv \frac{e^{-\tau/m}(1-e^{-\tau/\lambda m})^{g-1}}{\lambda m B(\lambda, g)}, \tag{20}$$

where $B(\lambda, g)$ is the 'complete' beta-function. (The genesis of Equation (20), which is a generalization of Yule's (1925) distribution is discussed by Jensen (1954); it is a member of the class of distributions considered by Kendall & Waugh (Kendall, 1952), namely those in which the variate is the sum of g variates each distributed as a negative exponential of, in general, arbitrary parameter. I am indebted to Dr D. G. Kendall for the Jensen reference.) When Equation (17) holds, Equations (18) and (20) evidently give rise to carrier distributions of the same functional form as themselves and the growth-rate constants are readily found to be given by:

Pearson Type III: $$\nu = \frac{1}{m}(2^{1/g}-1);$$

Distribution (20): $$\frac{1}{2} = \frac{\Gamma\{\lambda(1+m\nu)\}\,\Gamma(\lambda+g)}{\Gamma\{\lambda(1+m\nu)+g\}\,\Gamma(\lambda)}.$$

For the Pearson Type V we have

$$1 = \frac{2}{\Gamma(g)} \int_0^\infty m^g\, \tau^{-g-1} \exp(-\nu\tau - m/\tau)\, d\tau.$$

On putting $a = 2\surd(m\nu)$, $b = \surd(m/\nu)$, $\tau = be^{\xi}$ and rearranging:

$$1 = \frac{4m^g}{b^g\Gamma(g)} \int_0^\infty e^{-a\cosh\xi} \cosh g\xi\, d\xi,$$

whence $$4m^{g/2}\nu^{g/2} K_g\{2\surd(m\nu)\} = \Gamma(g),$$

where $K_g(a)$ is the modified Bessel function of the second kind (with the definition adopted by Watson (1952)).

Table 10 gives the estimates of the first (μ_1', $= a$) and second (μ_2, $=$ var τ) moments and the skewness (γ_1, $= \surd\beta_1$) and kurtosis (γ_2, $= \beta_2 - 3$) of the observations. The representative points of the distributions in the Pearsonian β_1, β_2 diagram (see, for example, Pearson & Hartley, 1954) are widely scattered; many fall near the Type III and Type V lines, but a few are far away in the Type IV area. I have not attempted to fit Type IV or Type VI distributions for the reason given above. Furthermore, the moments of some of the series can be accommodated to a Type VI only if its lower terminus lies above the origin. The arithmetic and geometric means for the Type III, and the harmonic and geometric means for the Type V, are sufficient statistics for the parameters g, m when the origin is taken as the lower terminus. The results of fitting both frequency functions by the method of maximum likelihood are given in Table 11. In only three series do both give a χ^2 probability below 0·05. We may take it that the distribution of τ can be represented for practical purposes by either a Type III or a Type V, though the χ^2 totals show that neither alone is satisfactory throughout. This conclusion is strengthened by the existence of association between the generation times of related organisms. If the τ of an organism falls in a given group, that of its sister is more likely to fall in the same group than it would be in the absence of association, and the true expectation of χ^2 is rather greater than n, the number of degrees of freedom.

Table 10. *Estimates of the first (μ_1', $=a$) and second (μ_2, $=var\ \tau$) moments and the skewness (γ_1) and kurtosis (γ_2) of the crude τ-distributions*

The unit of time is 1 min.

Series	No. of observations	μ_1'	μ_2	γ_1	γ_2
Ba 1	372	29·57	36·9	0·81	2·5
2	372	24·42	26·9	0·38	1·7
3	318	49·15	75·5	0·65	0·9
4	394	52·35	132·8	1·31	4·9
5	323	27·51	21·6	0·55	0·3
6	378	33·74	39·2	0·95	1·3
Cp 1	367	22·88	23·3	1·61	6·7
2	377	81·84	213·4	0·68	1·0
3	376	86·06	330·4	0·62	0·2
4	398	53·59	110·3	0·84	2·9
5	319	75·66	157·8	0·11	−0·2
Sf 1	364	30·21	18·8	0·83	1·1
2	442	26·12	12·0	0·27	0·2
3	446	27·21	20·5	1·21	8·1
Pp 1	365	31·08	27·3	0·72	1·4
2	329	38·11	28·7	0·39	−0·1

Table 11. *Frequency function parameters and goodness of fit, by maximum likelihood*

	Type III (Equation 18)					Type V (Equation 19)				
Series	g	m	χ^2	n	$P(\chi^2)$	g	m	χ^2	n	$P(\chi^2)$
Ba 1	24·2	1·22	3·85	11	0·97	23·9	678	14·3	11	0·22
2	20·8	1·18	28·9	10	0·0013	18·0	417	53·4	11	0·0000
3	32·0	1·53	3·08	5	0·69	32·2	1534	4·04	5	0·54
4	23·0	2·28	9·82	8	0·28	22·5	1124	15·6	8	0·049
5	35·3	0·779	6·39	8	0·60	35·6	952	5·48	8	0·71
6	30·7	1·10	22·9	11	0·018	32·0	1046	9·99	11	0·53
Cp 1	24·6	0·929	20·8	8	0·0077	25·9	569	15·3	9	0·082
2	32·0	2·55	7·85	10	0·64	32·1	2544	6·87	11	0·81
3	23·0	3·75	24·1	13	0·030	23·0	1898	18·3	12	0·11
4	26·2	2·04	19·7	7	0·0062	25·5	1313	21·2	8	0·0067
5	35·0	2·16	32·6	10	0·0003	33·6	2467	17·6	9	0·041
Sf 1	49·9	0·605	20·5	7	0·0047	51·3	1521	13·4	8	0·099
2	55·2	0·473	7·87	6	0·25	54·5	1397	5·26	6	0·51
3	36·8	0·739	11·90	8	0·16	35·7	945	21·2	9	0·012
Pp 1	36·3	0·857	8·74	10	0·56	36·8	1114	7·99	9	0·54
2	50·6	0·753	13·24	9	0·15	50·8	1898	10·7	10	0·38
Totals	—	—	242·2	141	0·0000	—	—	240·6	145	0·0000

In a few series I have also fitted the two distributions by the method of moments, with results not sensibly different from those given by the method of maximum likelihood. The labour of fitting (20) by the method of maximum likelihood is prohibitive; the moment fit, in the few cases tried, has been less good than that of Type III, the fitted curves being noticeably too unsymmetrical near the mode (Fig. 6).

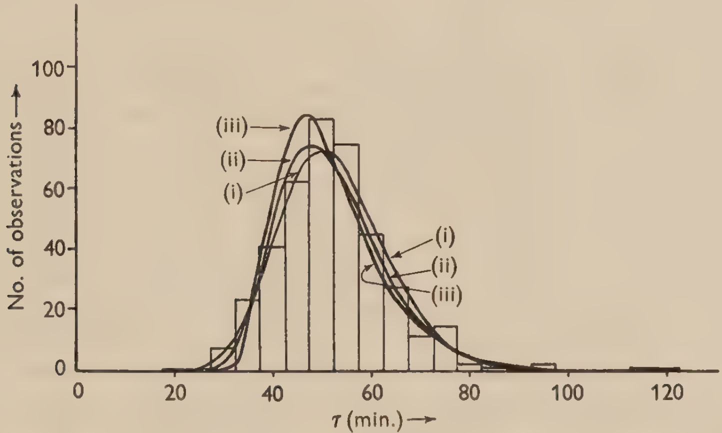

Fig. 6. Distribution of τ in Series Ba 4. Curve (i) Pearson Type III distribution fitted by maximum likelihood; (ii) Pearson Type V distribution fitted by maximum likelihood; (iii) Generalized Yule distribution (Equation 20) fitted by moments.

CONCLUSION

In broadest outline, the conclusion is very simple; there is positive association between the generation times of organisms which are closely related by descent. Where the association is unexpectedly weak, i.e. between mothers and daughters, a plausible explanation can be found by an examination of known features of the fission process. This kind of pattern is of course a commonplace in biology; the point of interest is that even among simple organisms chance alone does not determine the individual life-span. And some other properties of bacteria appear to be associated in the same way.

Eddy & Hinshelwood (1953) exposed washed cells of 'Bacterium lactis aerogenes' to the action of toxic substances (acid, *m*-cresol, acriflavine). They found that the organisms which happened to survive the treatment did not transmit their apparent resistance to their distant progeny; when the progeny were challenged in the same way, the proportion of survivors was the same as in the original culture. I have repeated some of these experiments under the microscope. It would not be appropriate to give details here, but the following result is typical: spores of *Bacillus cereus* were inoculated on to a culture chamber (Harris & Powell, 1951) and allowed to grow for 2·5 hr. Then a field was selected containing several groups of organisms, each group comprising the progeny of a single spore. The medium was exchanged for a glycine + hydrochloric acid buffer (pH 3) and after 5 min. the original medium was replaced. Most of the organisms were destroyed by the acid reagent; the survivors were identified by their resumption of growth:

Grouping of selected organisms	Survivors
11 groups of 2	2 organisms in 1 group
4 groups of 3	None
9 groups of 4	4 organisms in 1 group

A few experiments of this kind gave overwhelming evidence for association between sisters and cousins in respect of their resistance to the reagent; but the resistance is not propagated even through one overnight subculture. Similarly Hughes (1955*a*) found association in the sensitivity of related organisms towards antibiotics, though his object was to demonstrate differences rather than similarities.

There are indications that casual variations in some properties of bacteria may be inherited for many generations, certainly for more than the three covered in my experiments (e.g. Rogers, 1953; Hughes, 1955*b*). Hughes purports to show that the growth rate is so inherited. He isolated single cells from large and small colonies of *Escherichia coli*, and allowed them to grow for a fixed time. He found a high correlation between the size of the colonies so formed and the size of those from which the parent cells were isolated. This study, as it is reported, does not prove that the growth rate (or generation time) is inherited in the sense asserted, for two reasons. (i) Inheritance of mean size of organism could be responsible for at least part of the effect. The difference in size of single individuals would not necessarily be obvious under the microscope. (ii) Because growth is a multiplicative process, and the size of a colony doubles during a mean generation time, the size after a given period of growth from a single ancestor is critically dependent on the epoch of fission of the ancestor, and therefore on its age at the beginning of the growth period, and on any lag induced by the manipulations.

I think that Hughes's results do probably demonstrate the persistent inheritance of some property, but it is not necessarily the growth rate. Again, the variety of *Aerobacter cloacae* which I have used occurs in two forms, one of which has a thicker mucous coat than the other. Both are to be found in ordinary cultures, and single cells of either may transmit their character through ten or more generations, after which reversions to the other form become frequent.

We are, I think, now justified in seeking the beginnings of a working hypothesis to explain the variability of individuals. No other class of organisms offers nearly so good a chance of solving this universal problem.

'An organism tends to keep its equilibrium amid the bombardment of aimless forces which beset it, to regain equilibrium when it has been disturbed, and to pursue a sequence of actions dictated by the need of keeping all its interdependent parts constantly renewed, their structure intact.' (Langer, 1953). The very general phenomenon of variability with regression has been given verbal expression in many ways; for bacteria, Langer's is precisely apt, because to them Brownian motion—'the bombardment of aimless forces'—is an immediate and effective reality.

A unicellular organism may contain 10^{10} or 10^{11} atoms, and of these a quite small fraction will be arranged in specialized ways to form molecules of catalysts or growth factors. Some few will be atoms of heavy metals, essential but required in only exiguous amounts. Thus Haldane (1954) quotes values of about 10 molecules/sec. for the rate of synthesis of some growth factors; the complement of catalase in most bacteria includes 10^4 iron atoms or less (Herbert & Pinsent, 1948); and the minute amount of molybdenum (perhaps 100 atoms/cell) necessary and sufficient to enable *Azotobacter chroococcum* to assimilate elementary nitrogen is well known. The visible Brownian motion of a bacterium suspended in a liquid medium is mostly caused by water molecules, and it is therefore evident that the acquisition of rare atoms or molecules from the medium must be a highly erratic process. Their internal chemistry, too, cannot but be equally erratic. The fewer the atoms or molecules concerned, the greater the relative fluctuation in their number; the slower the reactions in which they are built up and broken down, the more persistent and widespread the effects of that fluctuation. The passage of molecules into the cell, and their reactions with others already present, will be subject to chance effects of two kinds, the first depending on their position in space (becoming diffusion on the large scale), the second on their orientation and thermal energy at critical moments (becoming reaction velocity on the large scale). Transient deviations from the mean rates and concentrations will be frequent, and no doubt sometimes so large as to be reflected in the subsequent behaviour of the organism because they overtax the self-regulating mechanisms, or alter the response to an inimical environment. Occasionally they may be lethal. But we can well admit that the organism is not entirely at the mercy of molecular accidents, and that so relatively simple a creature may be able to resort to more than one stable or metastable state if it is driven to do so. Eddy (1953*b*), discussing the work of Eddy (1953*a*) and Eddy & Hinshelwood (1953) on the survival of organisms treated with acid, adumbrates this same idea; he writes of 'a chance conjunction of events', 'complex fluctuations in the concentrations of many molecular species', as determining the fate of particular individuals. We may perhaps hope in the future to analyse the determinants of generation time fluctuation, or at least to distinguish two in a quantitative way: (i) the immediate chance effect resulting from molecular accidents, as in the hypotheses of Kendall and Rahn; (ii) the inherited tendency whose primary cause may again be accident, but which in any case derives from ancestors more or less remote.

Mr E. C. Fieller (private communication) has suggested the possibility of representing the correlation between generation times as resulting from a process of 'autoregression'. If τ_1, τ_2 are generation times of mother and daughter, for example, we might write

$$\tau_2 = a\tau_1 + bx, \tag{21}$$

where a and b are constants, $a+b=1$, and x is a random variable. This scheme, or some elaboration of it, could evidently be made to bear a close analogy to the ideas considered above. In its simplest form (21), it implies that the mother-daughter correlation $\rho(H)$ is a, and the grandmother-granddaughter (say $\rho(H_2)$) is a^2. It would not be difficult to test the hypothesis that

$$\rho(H_2) = \{\rho(H)\}^2.$$

In this paper I have treated the observed τ as representative of a secondary process, keeping in mind the scheme of relations of Figs. 1, 4 and 5. I have already given reasons for the legitimacy of this viewpoint, but at best they should only be taken to apply to unicellular organisms. Consider a p-tree as in Fig. 4 or Fig. 5 with associated δ of small variance but of mean value several times the mean τ_p, say about $n\tau_p$. Then the 'organisms' produced at o-fission will consist of about 2^n cells, which will grow to about 2^{n+1} before the next o-fission. We can regard multicellular organisms as being formed in this way. But, remembering the additive property of independent variances, it is clear that even a moderately regular morphology implies close coupling of the τ_p and δ processes. If, furthermore, the groups of cells are indeed organisms *sensu stricto*, their τ will have a biological significance independent of the δ or of any 'corresponding' τ_p. I think therefore that the generation times of multicellular organisms are as much deserving of study as the those of unicellular, especially as our knowledge of intercellular organisation is so little developed.

Mr T. W. Pearce and Mr J. M. Scott assisted in the maintenance of cultures and of the apparatus, and shared in the observations. Much of the computation was carried out by Mr R. Ash.

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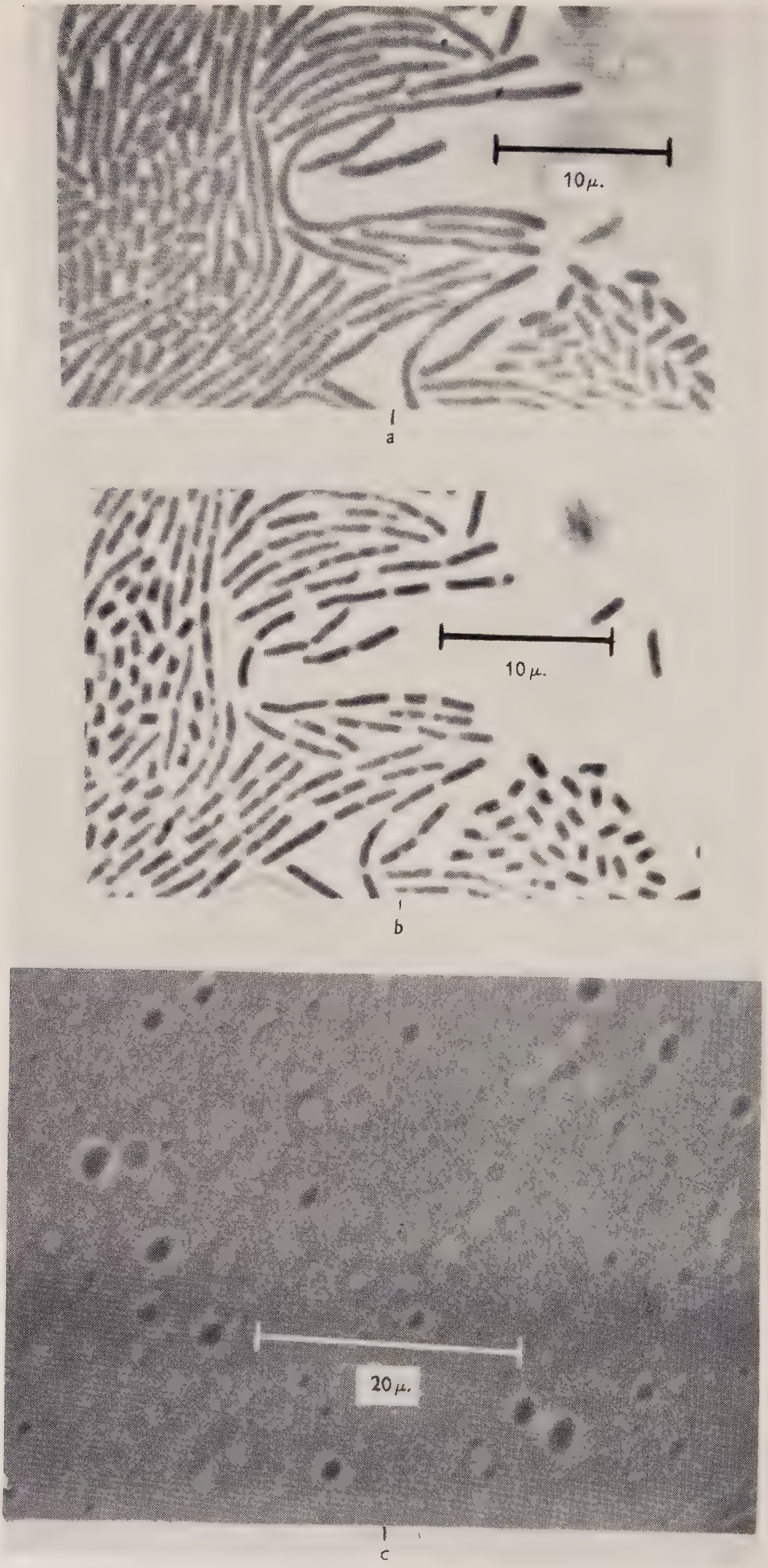
10μ.
a
10μ.
b
20μ.
c

E. O. Powell—The pattern of bacterial generation times. Plate 1

(*Facing p.* 417)

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EXPLANATION OF PLATE

(*a*) *Escherichia coli*. Long organism formed by action of *m*-cresol. Phase contrast.

(*b*) *E. coli*. Same field as (*a*). Organisms plasmolysed by treatment with 5 % NaCl. Phase contrast.

(*c*) Structure of Cellophane. The focal plane lies within the thickess of the membrane. Taken with a variable phase-contrast microscope adjusted for maximum contrast.

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Observations on *Phaeodactylum tricornutum*

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SUMMARY: *Phaeodactylum tricornutum* Bohlin was isolated from a marine tank at Woods Hole, Mass., U.S.A. It appeared to be identical with the Plymouth strain of '*Nitzschia closterium* forma *minutissima*'. Cells were of two characteristic types, oval and fusiform, each of which remained constant for many cell divisions in clonal culture. Triradiate cells arose rarely as atypical forms of the fusiform variety. Oval cells could arise as endospores within a fusiform cell. The transition from oval to fusiform was also studied, but details of the life cycle remain to be worked out. Electron micrographs showed the fusiform cells to be devoid of any organized siliceous structure, in agreement with previous observations. However, the oval cells were seen to possess a silica valve of a pennate diatom type, resembling those of the genus *Cymbella*. Only one valve was present on each cell, the remainder of the cell wall being unsilicified. The valve was 6·2 μ. long, was equipped with a raphe, and was perforated by pores arranged in 60 striae. Oval and fusiform cells both contained approximately the same amount of silica (0·4–0·5 % dry weight). In each case, most of this silica could be recovered as a particulate fraction resistant to digestion in hot nitric acid. The silica obtained from oval cells was in the form of diatom valves, whereas that from fusiform cells consisted of irregular particles clearly not derived from broken silica walls.

Mucilaginous capsular material, soluble in hot water, represented 16 % of the dry weight of oval cells; it was absent from fusiform cells. Acid hydrolysis and paper chromatography indicated xylose, mannose, fucose, and galactose as components of the capsule.

Allen & Nelson (1910) isolated a small marine organism, which they referred to as '*Nitzschia closterium* W. Sm. forma *minutissima*'; unfortunately they did not publish any formal description or illustration of it. The fusiform cells resembled in shape those of the diatom *N. closterium* (Ehrenberg) W. Sm., but the dimensions were smaller. Cultures of this smaller organism isolated by Allen & Nelson (1910) have been maintained at Plymouth Marine Laboratory, Devon, England, and distributed to laboratories around the world. Since it grows rapidly in a wide range of temperature and illumination and in many different nutrient media, much of the fundamental work published on marine diatoms has been carried out with this strain. In addition, it has been extensively used as food for raising a variety of marine animals. Some unusual features exhibited by this organism have prompted further studies to elucidate its nature and affinities.

Barker (1935) observed that a contaminated culture of the Plymouth strain, which had been maintained at the Hopkins Marine Station in California for several years, contained spindle-shaped cells, oval-shaped cells, and cells shaped like three-pointed stars. In the course of isolating bacteria-free cultures, he observed a tendency for the spindle-shaped cells to become less numerous on successive transfers to fresh agar while the number of oval cells increased. When grown in liquid media, on the other hand, a gradual reversion to spindle-shaped cells occurred. Triradiate cells were only rarely observed. Barker confirmed that these various cell forms all belonged to a genetically pure line. In 1942 Wilson & Lucas reported that triradiate forms of this organism arose independently in cultures maintained in Plymouth and in Hull. In a detailed illustrated account of this unusual organism, Wilson (1946) reported the presence of fusiform, triradiate and cruciform cells, and observed that both fusiform and triradiate cells sometimes gave rise to ovals. The ovals so formed either divided to produce more ovals or grew two or three arms to form fusiform or triradiate cells, respectively. Unlike the other cell types, the oval cells tended to produce mucilaginous capsules. Wilson did not observe auxospores, and stated that they were therefore not necessary for the elongation of the cells. The cell walls were so weakly silicified that he could not distinguish the valve markings characteristic of the Nitzschioideae. He considered the triradiate form to be identical with an organism of doubtful affinities, *Phaeodactylum tricornutum* Bohlin, 1897. Electron micrographs of fusiform and triradiate cells of the Plymouth strain showed no evidence of a characteristic diatom structure in the walls (Hendey, 1954; Bourrelly & Dragesco, 1955). In the material examined by these workers there were evidently few or no oval forms present, since these were not illustrated. In the course of physiological studies of the factors responsible for the changes of form of this organism, we prepared electron micrographs of oval as well as of fusiform cells. It immediately became apparent that *P. tricornutum* is an even more unusual and interesting organism than we had hitherto suspected. The structure of the walls of oval cells, as well as chemical and physiological differences between oval and fusiform cells, are described in this paper.

METHODS

In September 1956, we observed fusiform cells of *Phaeodactylum tricornutum* in a large outdoor tank behind the Woods Hole Oceanographic Institution, containing sea water enriched with a commercial fertilizer. By serial streaking of the cells on enriched sea-water agar plates we isolated the organism in bacteria-free culture. We also obtained from Dr M. Droop (Marine Station, Millport, Scotland) two other pure cultures of *P. tricornutum* for comparison: Millport No. 14, isolated by Droop in 1951 from a rock pool on the island of Segelskär, off the south coast of Finland, and Millport No. 15, the Plymouth strain. When the Woods Hole strain and the two European strains were grown in separate samples of the same batch of medium, they appeared identical. For the studies reported in this paper we used only the Woods Hole strain.

Two basal culture media were used:

(1) Enriched sea water, which consisted of nine volumes of sea water to one volume of a supplementary mineral solution; the final concentrations of added minerals were $Ca(NO_3)_2 . 4H_2O$, 0·01 %; K_2HPO_4, 0·002 %; 0·5 p.p.m. Fe; 0·3 p.p.m. Zn; 0·1 p.p.m. each of B, Co, Cu, Mn, Mo; $Na_2SiO_3 . 9H_2O$ to give a

Si concentration of 5 p.p.m. In some cases Tryptone (Difco) was added at a concentration of 0·1 %.

(2) Artificial sea water, which consisted of NaCl, 2·0 %; $Ca(NO_3)_2.4H_2O$, 0·05 %; K_2HPO_4, 0·01 %; $MgSO_4.7H_2O$, 0·01 %; trace elements and silicate as in (1). For solid media, agar was employed at a concentration of 1·0 % (w/v). The cultures were constantly illuminated with white fluorescent lights (300 f.c.) and maintained at 21°. Aeration of liquid mass cultures was provided by a slow passage of air filtered through sterile cotton wool.

Samples of dry organisms were analyzed for major constitutents as follows: (1) Micro-Kjeldahl N determinations ($\times 6{\cdot}25 =$ protein); (2) total ash and total silica; (3) silica in valves after digestion with hot concentrated nitric acid. In addition 100 mg. samples of dry organisms were extracted by: (1) successive methanol extractions; (2) cold dilute hydrochloric acid, pH 3; (3) water at 100°. Ash and silica were determined in the cell residues remaining after these extractions. The methanol extracts (1) were pooled, the solvent removed by evaporation, and the solid residue, comprising various pigments and lipids, was dried in a vacuum desiccator and weighed. The cold aqueous extract (2) contained inorganic salts and soluble carbohydrates including leucosin. Free and combined hexoses were determined quantitatively by the anthrone reaction and were expressed as glucose equivalents. Two successive water extractions at 100° (3) removed the capsular material from the cells. The extracts were concentrated to 5 ml. and poured into 15 ml. ethanol. The flocculum which formed was removed by centrifugation, washed, dried and weighed.

Paper chromatographic analysis of the capsular material was carried out as follows. A sample of the dried material (5 mg.) was heated in a sealed tube with 1·0 ml. of 0·7 % H_2SO_4 for 6 hr. at 105°. The hydrolysate was neutralized with $BaCO_3$, and the supernatant fluid evaporated to dryness and redissolved in one drop of water. This extract was chromatographed on paper for 16 hr. together with reference sugars, using either of two separate solvent mixtures: (*a*) butanol:H_2O:acetic (4:1:1), or (*b*) phenol:H_2O (4:1) in ammonia vapour. The chromatograms were dried, sprayed with 1 % (w/v) aniline phthalate, and developed for 5 min. at 100° .

Silicon determinations were carried out as follows. Material such as acid-cleaned valves, or the ash from dried cells or extracted cell residues, was fused with excess sodium carbonate in platinum crucibles. The flux was dissolved in water, and the silicon in solution was determined colorimetrically by the ammonium molybdate reaction (Lewin, 1954).

In preparing material for electron microscope observations we used whole cells, walls from cells burst by rapid transfer into distilled water, and silica valves prepared by digesting the cells with hot concentrated nitric acid. An R.C.A. Model EMU electron microscope was used.

RESULTS

Character of colonies on an agar surface

The colonies of *Phaeodactylum tricornutum* which formed from cells streaked on agar were mainly of two different types. Those composed of fusiform cells were dark brown, rounded, with smooth margins, and showed a satiny iridescence due to the parallel alignment of the cells. Colonies of oval cells were more diffuse, with irregular edges where individual cells migrated over the surrounding agar, and of a lighter shade of brown since the cells were separated from one another by colourless mucilaginous capsules. These two types of colonies could be readily distinguished with a binocular dissecting microscope, and, after growth for 2 or 3 weeks, even with the naked eye. It was possible also to recognize colonies, initially comprising only fusiform cells, but later giving rise to oval cells, which began to spread over the agar from one or more sites at the edge of the colony. The fact that oval cells could creep over a surface in this fashion, as do many species of pennate diatoms, suggested that cells of this type might possess a raphe, as do all motile diatoms.

Observations on the life cycle

Wilson (1946) attempted to follow the life cycle of his organism by examination of cells from liquid cultures. In cultures originating from oval cells, he noted that cells with short arms appeared, and that the average length of the arms increased in successive transfers. Although normally such cells bore two arms, he occasionally observed the origin of triradiate or other variant forms. We followed the development of individual cells on agar by drawing them at daily intervals, and observed:

(*a*) Oval cells divided to form oval cells (confirming Wilson's observations).

(*b*) Fusiform cells divided to form fusiform cells of similar length and form (confirming Wilson's observations).

(*c*) Inflated fusiform cells, approximately 25μ. long, produced a pair of ovals (cf. Wilson, Fig. 2) by division of the protoplasm within the central enlarged region of the cell. These oval cells sometimes divided again and were ultimately released by rupture of the parent wall. This observation did not bear out Wilson's supposition that oval cells arose from fusiform cells by unequal division.

Liquid and agar cultures also occasionally contained spherical auxospores, naked vacuolate protoplasts 6–12μ. in diameter, which were usually attached to an empty wall of a fusiform cell. All our attempts to follow the fate of individual auxospores on agar have so far proved unsuccessful.

Among 64 subclones which originated from single colonies on agar and were grown in liquid medium, one was found in which the cells were initially all triradiate. Since this form was believed to be atypical, the following experiment was carried out. The culture was allowed to age until abundant oval cells had been produced. It was then streaked on agar, and after 10 days 100 colonies of oval cells were isolated and transferred into liquid medium 1. When examined after 28 days, these cultures contained only oval cells.

Transfers were then made into medium 1 + Tryptone, which had been found to promote the production of armed cells. Ten days later all the cultures had produced abundant fusiform cells. Since no triradiate forms were seen, this form evidently does not persist beyond the intermediate stage of the oval cells. This confirms observations by Wilson (1946) and by Droop (Hendey, 1954), and supports the view that triradiates are not a normal stage of the life cycle. These studies are being continued with a view to determining what factors may be responsible for the changes in cell form and whether a sexual cycle may be involved.

Cell walls

The oval cells of *Phaeodactylum tricornutum* are approximately 8 μ. long and 3 μ. wide; the fusiform cells extend to a length of 35 μ. Intact oval and fusiform cells, and cell membranes of both cell types prepared by bursting the cells in distilled water, were examined with the electron microscope. No perforated valves were seen on any of the intact fusiform cells (Pl. 1, figs. 1, 2), nor on the fusiform membranes (Pl. 1, figs. 3, 4), confirming the observations of Hendey (1954) and of Bourrelly & Dragesco (1955). However, the oval cells were seen to possess a valve of opaque material, presumably silica, of a typical pennate diatom structure (Pl. 2, figs. 5, 7). This valve was more clearly distinguishable in preparations of cell membranes freed from other cell material, and appeared as a silicified area on one side (Pl. 2, figs. 6, 8).

A typical diatom cell regularly possesses a silica frustule, composed of two valves and connecting bands, which completely encloses it in a silica shell. In both cell types of *Phaeodactylum tricornutum* the cell membranes appeared to be composed of a pair of overlapping parts, most readily perceived at the ends of cells seen in side view (Pl. 1, fig. 2 and Pl. 2, fig. 9). These evidently correspond to the epitheca and hypotheca of a typical diatom. The unsilicified portions of the cell membranes were transparent to the electron beam, pliable and structureless, except for faint indications of a fibrous nature. A unique feature of the oval cells of *P. tricornutum* was the presence of only one valve covering only part of the cell surface (Pl. 2, fig. 9). This was the case in over 90 % of the cells examined; in the remainder, for one reason or another, no valve could be seen.

When oval cells were digested with nitric acid, the cell contents and membranes disappeared and only the valves remained, thereby confirming their siliceous nature. The 'cleaned' valves were *c.* 6·2 μ. long by 1·6 μ. wide, with a raphe, a central and polar nodules, and 60 rows of pores or striae (equivalent to 95 in 10 μ.). The apical axis of the valve was slightly curved producing a shape characteristic of the genus *Cymbella* (Pl. 2, fig. 10). Identical silica valves were also found in the two Phaeodactylum strains sent to us by Droop. Though the valve markings of the strain used here are so fine that it would be impossible to distinguish them with a light microscope, we considered that the valves themselves should be detectable without recourse to electron microscopy. Once we knew what to look for, we extracted the pigment from some oval cells with methanol, stained the cells with toluidine blue, and mounted them in Hyrax. When we examined them under oil immersion we could make out in some cells the dark line of the axial area on the surface.

Chemical analyses of fusiform and oval cells

By inoculating tubes of liquid medium with oval and with fusiform cells from selected colonies of *Phaeodactylum tricornutum* on agar, clones of the two cell forms were isolated. Mass cultures of each were grown in 2 l. Erlenmeyer flasks under conditions of constant temperature, light, and aeration. After incubation for 16 days, aeration was stopped. The oval cells, which grew as clumps, settled within a few minutes to the bottom of the flask, whereas the fusiform cells remained in suspension for some hours. The cells were concentrated and separated by centrifugation. The fusiform cells packed to form a smooth pasty mass, occupying only half the volume taken up by the gelatinous mass of oval cells. Microscopic examination revealed among the fusiform cells less than 1 % of ovals. The yields from 1·5 l. culture medium in one experiment are summarized in Table 1.

Table 1. *Yield of* Phaeodactylum tricornutum *from 1·5 l. of culture medium*

	Oval cells	Fusiform cells
Packed cell volume (ml.)	5·8	2·7
Total number of cells	$2{\cdot}15 \times 10^{10}$	$2{\cdot}17 \times 10^{10}$
Dry weight (mg.)	431	439

Table 2. *Chemical composition of oval and fusiform cells of* Phaeodactylum tricornutum

	Organisms	
	Oval	Fusiform
	% of dry wt.*	
Lipid	24	34
Protein	34	41
Carbohydrate		
Soluble in cold dilute acid	3	2
Soluble in hot water, precipitated with ethanol (capsular material)	16	0
Miscellaneous		
Soluble in cold dilute acid (not carbohydrate, not ash)	10	10
Soluble in hot water, not precipitated with ethanol	3	2
Ash	13	12
Total	103	101

* Averages of 2–3 determinations

The cells were analyzed as described on p. 420 (Methods), and the results shown in Table 2. Approximately 16 % of the dry weight of oval cells consisted of a mucilaginous material, soluble in hot water, and corresponding to the cell capsules which stain metachromatically with toluidine blue. Paper chromatograms of the hydrolysate of this substance indicated the presence of xylose, mannose, fucose and galactose. A mucilaginous fraction was not present in the fusiform cells, which lacked capsules, but which contained higher percentages of lipid and protein than the ovals.

Silica determinations were carried out on samples of dried cells of each type which had been rinsed with cold dilute acid, washed, dried and ashed. Both oval and fusiform cells contained approximately the same amount of silica (0·4–0·5 % of dry weight). This was unexpected, since no silica walls have ever been observed in the fusiform cells. Separate samples of oval and fusiform cells were digested with hot concentrated nitric acid. After this treatment, a small amount of white flocculent material remained floating on the surface of the nitric acid in each case. No silica valves were present in this flocculum. In the case of the oval cells, there also always remained a white precipitated deposit, which proved on chemical analysis to contain most of the cell silica (0·3–0·4 % of dry weight), and which consisted of diatom valves as revealed by the electron microscope. In two out of four analyses of fusiform cells, a white precipitate was also obtained after nitric acid digestion. This likewise proved to contain most of the cell silica (0·3–0·4 % of dry weight), but when examined with the electron microscope it appeared to be composed only of structureless debris, comprising irregular particles of various sizes, plainly not derived from silica walls.

DISCUSSION

It seems to be the opinion of most workers who have studied *Phaeodactylum tricornutum* that the oval and the fusiform cells are the typical prevalent stages in the life cycle. With prolonged cultivation on an agar surface the oval cells predominate, apparently being favoured since they have some ability to spread over the agar surface. Barker (1935) and Droop (see Hendey, 1954) have observed the transition to a predominance of oval cells on solid media. On the other hand, on prolonged cultivation in a liquid culture the fusiform cells predominate. Apparently they are at an advantage since in an actively growing culture they are more buoyant, whereas the oval cells tend to associate in clumps and sink to the bottom. Both Barker and Droop have reported a tendency for the formation of fusiform cells in liquid culture.

The triradiate cells have been the only form hitherto readily recognizable in samples of littoral water from nature. This is evidently because the fusiform cells might be mistaken for shortened *Nitzschia closterium*, and the ovals cells, when noticed at all, would be classified as minute, unidentifiable pennate diatoms. Triradiate forms originate rarely in the laboratory, but under certain conditions, as in static cultures, such cells may be favoured by selection, perhaps because of a decreased rate of sinking, and may overgrow the other forms. In addition to triradiates, cruciform cells (Wilson, 1946), curved cells, and other abnormal forms have occasionally been observed. When such a morphological abnormality arises, it reproduces clonally, forming daughter cells of the same shape as the parent cell, since cell division takes place in a longitudinal plane. The cell membrane is evidently inelastic enough to maintain its characteristic shape through successive cell divisions. However, variants of this sort tend to persist only until they produce oval cells, since these in due course typically give rise to normal fusiform cells.

Some data on the chemical composition of '*Nitzschia closterium* forma *minutissima*' have been reported by previous workers. Bidwell, Krotkov & Reed (1952) analyzed for free and bound sugars in cells of the triradiate variety. They found approximately 0·3 mg. ethanol-soluble sugars (glucose, fructose, sucrose) in 10^{10} cells. According to our calculations these free sugars would represent approximately 0·15 % of the dry weight. These workers also reported 6·4 mg. carbohydrate from the ethanol-insoluble residue, which we estimate would represent approximately 3·2 % of the dry weight. After acid hydrolysis, glucose, representing 2·5 % of the dry weight, was found to be the main constituent of this fraction. This glucose most probably came from leucosin, a water-soluble polymer of glucose characteristic of the Chrysophyta and reported from *Phaeodactylum tricornutum* by Parke (Hendey, 1954). In our analyses, leucosin could account for the 2–3 % of soluble carbohydrate in the cold dilute acid extract, a value in agreement with that calculated from the data of Bidwell, Krotkov & Reed (1952). Probably there were few or no oval cells in the material analyzed by these workers, since they did not report insoluble polysaccharide sheaths. Ketchum & Redfield (1949), using an empirical formula, calculated the proportions of protein, carbohydrate and lipid in the same organism, based on the R value (degree of reduction) and on the percent protein Their figures indicated 42 % protein, 25 % lipid, 33 % carbohydrate in the organic fraction, and an ash residue representing 19·5 % of the dry weight. Expressed on the same bases, our values were, respectively, 39, 28, 22 and 13 % for ovals, and 47, 39, 2 and 12 % for fusiform cells.

Harvey (1955) reported that about 1 % of the dry weight of the *Phaeodactylum tricornutum* he examined was silica. In the present study, the washed cells of both forms of our *P. tricornutum* were found to contain approximately 0·4 % silica on a dry-weight basis. This is present in the oval cells largely as silica valves. It is not known in what chemical condition the silica exists in the fusiform cells, since no recognizable structures remained after digestion with concentrated nitric acid. In comparison with other diatoms, Phaeodactylum is very weakly silicified, and enough silicon dissolves from Pyrex glass vessels in alkaline culture media to fulfil its meagre requirements. Harvey (1955) reported that heavy crops of *P. tricornutum* were produced in enriched sea water in hard-glass vessels without added silicate or silica sol, and we observed no difference in the rate of growth of oval or fusiform cells in artificial sea-water media, whether silicon was added or not. Analyses and physiological studies of this organism have been extensively quoted as representative of the diatoms. Present evidence seems to indicate that *P. tricornutum* is a most atypical diatom; its taxonomic position will be discussed in the following paper (Lewin, 1958).

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EXPLANATION OF PLATES

PLATE 1

Electron micrographs of *Phaeodactylum tricornutum*. Each scale line represents 2μ.

Fig. 1. Fusiform cell.
Fig. 2. Fusiform cells.
Fig. 3. Membrane from fusiform cell.
Fig. 4. Membranes from fusiform cells.

PLATE 2

Electron micrographs. Each scale line represents 2μ.

Fig. 5. Oval cells.
Fig. 6. Membranes from oval cells.
Fig. 7. Oval cell.
Fig. 8. Membrane from oval cell.
Fig. 9. Oval cell, side view.
Fig. 10. Silica valves of oval cells.

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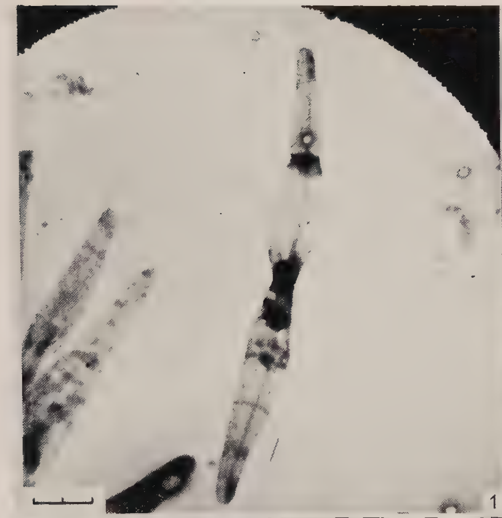

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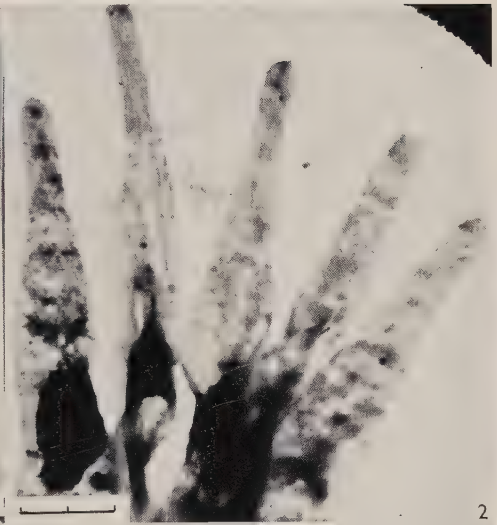

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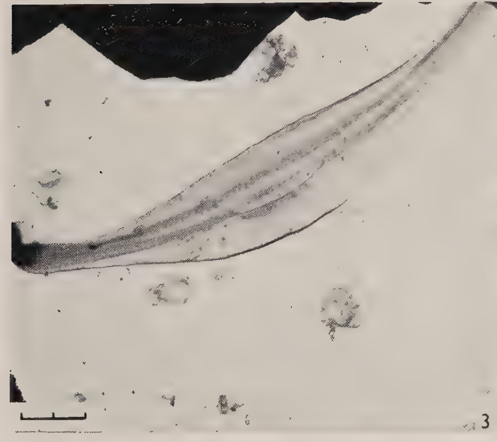

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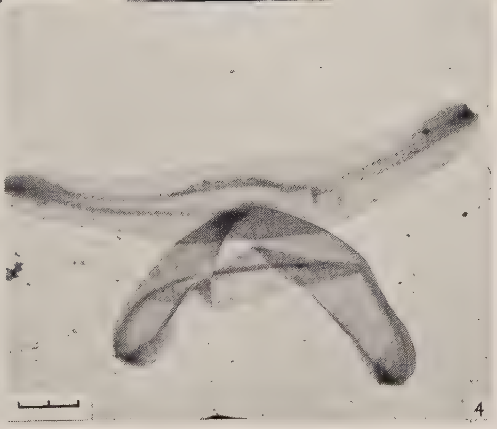

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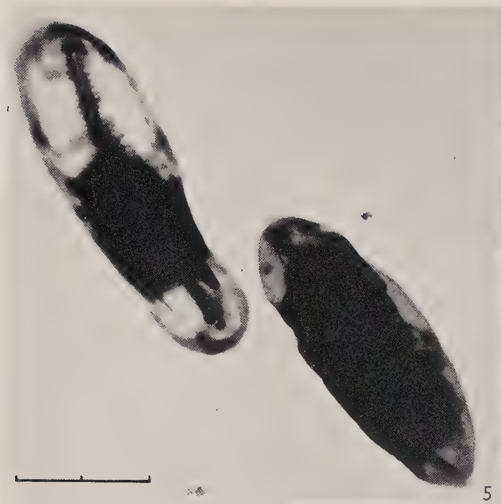
J. C. LEWIN, R. A. LEWIN AND D. E. PHILPOTT—*PHAEODACTYLUM TRICORNUTUM*.
PLATE 1

(*Facing p.* 426)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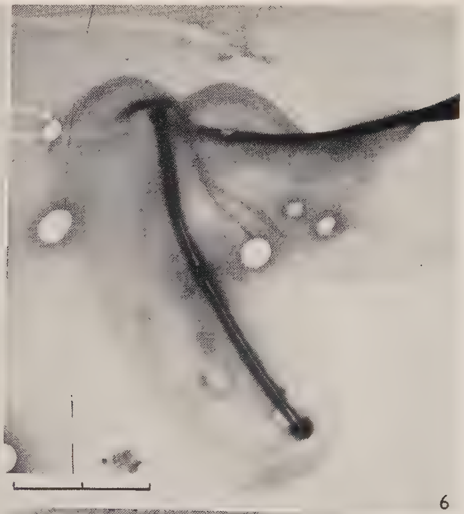
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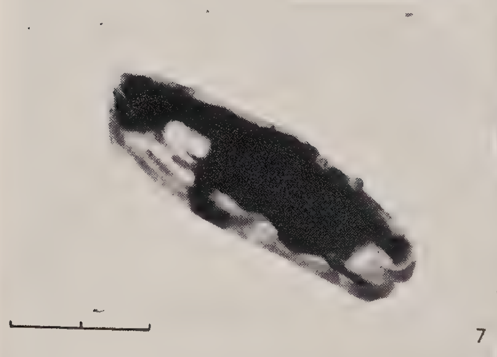
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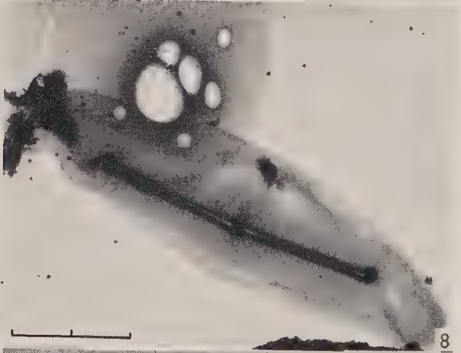
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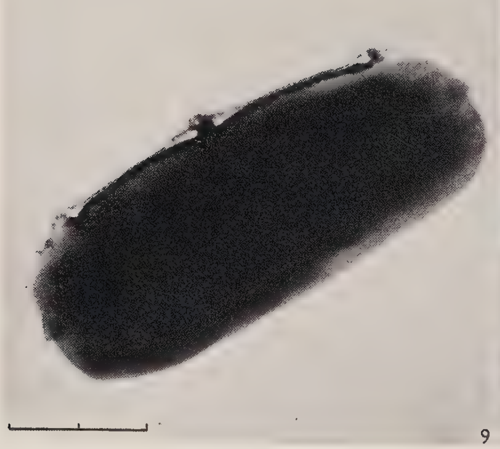
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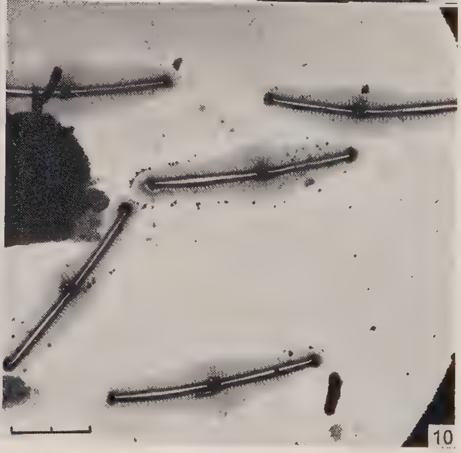
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J. C. LEWIN, R. A. LEWIN AND D. E. PHILPOTT—*PHAEODACTYLUM TRICORNUTUM.* PLATE 2

Lewin, J. C. (1958). *J. gen. Microbiol.* **18**, 427–432

The Taxonomic Position of *Phaeodactylum tricornutum*

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SUMMARY: A revised taxonomic description of *Phaeodactylum tricornutum* Bohlin is presented, with special emphasis on the details of the silica valve, a structure hitherto unobserved. The features distinguishing *P. tricornutum* from *Nitzschia closterium* (Ehr.) W. Sm., with which it has frequently been confused, are summarized. In view of the unique characters of *P. tricornutum*, it is proposed to establish within the Bacillariales a new suborder, the Phaeodactylineae, to which *Phaeodactylum* is assigned as the only known genus.

In 1897 Bohlin described a new genus of unicellular algae, *Phaeodactylum*, with a single species, *P. tricornutum*. The organism was stellate with three narrow arms, possessed yellow-brown chromatophores and was weakly silicified. On the basis of the chemical nature of the cells and their characteristic plane of division, parallel with that of the arms, Bohlin considered that *P. tricornutum* was closely related to the diatoms. Wilson (1946), in a study of the various cell forms of '*Nitzschia closterium* W. Sm. forma *minutissima*', recognized the triradiate form of this organism as identical with *P. tricornutum* Bohlin. Since Hendey (1954) found no evidence for siliceous structures in the cell walls of this organism, he felt that it could not be classed as a diatom, and the presence of leucosin, as shown by Parke (recorded by Hendey, 1954), suggested to him a possible relationship to the Chrysophyceae. On the basis of the absence of movement, the absence of a raphe, and the absence of siliceous structure, Bourrelly & Dragesco (1955) also concluded that *P. tricornutum* could not be regarded as a diatom, but was a 'primitive Chrysococcalian'. Since the oval cells of *P. tricornutum* have now been shown to possess a silica valve (Lewin, Lewin & Philpott, 1958), it seems necessary to describe the organism more precisely on this basis, and to assign it to an appropriate taxonomic position. Though the specific epithet *tricornutum* might appear inappropriate, having been based on an atypical form, it has to be retained according to the rules of the International Code of Botanical Nomenclature (Lanjouw *et al.* 1956).

NEW DESCRIPTION

The emended description of the species is as follows:

Phaeodactylum tricornutum Bohlin emend.

Unicellular, normally with one parietal brown chromatophore in central region. Two typical forms of cells. (*a*) Ovate, 8 μ. long × 3 μ. wide, one silica valve per cell. Cells may be slowly motile, or immobile in mucilaginous clumps. (*b*) Fusiform, up to 25–35 μ. long, with two more or less blunt, slightly bent arms.

Silica wall absent. Non-motile. Rarely variants occur (triradiate, cruciform, lunate, etc.) which tend to persist in clones.

Valve weakly silicified, with a thin ragged margin; 6·2μ. long by 1·6μ. wide. Girdle bands absent. Valve flat, linear lanceolate, slightly curved, symmetrical about the transapical plane (as in *Cymbella* spp.); one edge convex, the other more or less straight with a median bulge. Ends broadly rounded but not capitate. Raphe slightly curved. Central area small, frequently with a pore on the side of the bulge. Striae approximately 60 (equivalent to 95 in 10μ.), mostly transverse but radiating in region of bulge. Puncta 2–6 per stria. (These details are revealed by electron microscopy. Under the light microscope it is just possible to detect the axial area, but no other features of the valve can be distinguished.)

CLASSIFICATION

Phaeodactylum tricornutum should undoubtedly be assigned to the Chrysophyta, since the cells possess golden-brown chromatophores, store leucosin and oil, and develop a silica wall at certain stages. The genus exhibits sufficient diatom characteristics to justify its inclusion within the class Bacillariophyceae. Among these features are the following: vegetative division longitudinal (usually not by formation of endospores), silica valve of a pennate diatom type, gliding movement of the oval cells, and auxospore formation.

The taxonomic position of *Phaeodactylum tricornutum* within the Bacillariophyceae is not readily apparent. Since the valve structure so closely resembles that of *Cymbella* spp. it might seem desirable to place the genus within the family Cymbellaceae. On the other hand, each oval cell possesses only one valve, and this feature distinguishes *P. tricornutum* from diatoms of all existing suborders. It is felt that this character, and the total absence of a siliceous wall from cells of the fusiform phase, provide sufficient justification for the formation of a new suborder. In the same way, various members of the Achnanthaceae, although their raphidate valves indicate close affinities to certain genera of the Biraphidineae, have been set apart as a separate suborder, the Monoraphidineae, on the basis of possessing dissimilar valves. It is therefore proposed that a new suborder, the Phaeodactylineae, be established. The taxonomic position of *P. tricornutum* would then be as follows:

Phylum	Chrysophyta
Class	Bacillariophyceae
Order	Bacillariales (Hendey, 1937)
Suborder	Phaeodactylineae (nov.subord.). Unlike all other diatoms hitherto described, the cells do not possess more than one silicified valve, which, when present, only partially covers the cell. Cellulae valva unica, cellulam partim tegenti, instructae, vel nudae.
Family	Phaeodactylaceae (nov.fam.). Characteribus subordinis Phaeodactylineae. The only family of the suborder.

Genus Phaeodactylum (Bohlin, 1897). The only genus. Cells existing in two typical forms: motile oval cells which possess a silica valve, and non-motile fusiform cells lacking a silica valve.
Species *P. tricornutum.* The only known species. (Description p. 427.)

Clarification of the distinctions between Phaeodactylum tricornutum *Bohlin and* Nitzschia closterium (*Ehr.*) *W. Sm.*

Over the past fifty years the organism known as '*Nitzschia closterium* forma *minutissima*' has been widely used in investigations of various aspects of physiology and biochemistry of diatoms and for studies of marine plankton. It is obvious now that a most atypical diatom was selected. *Phaeodactylum tricornutum* is apparently not even a normal component of marine phytoplankton, since it has usually been reported from intertidal rock-pools or sea-water tanks. It therefore seems unjustifiable to extend conclusions drawn from studies with this alga to diatoms in general or to phytoplankton. As a further complication, physiologists using the Plymouth strain often dropped the designation 'forma *minutissima*', and referred to it in publications simply as '*N. closterium*'. Since a true *N. closterium* exists, which is very different from *P. tricornutum* (see Table 1), it is often difficult to be sure which of the two organisms was being studied. As far as can be determined, the Plymouth strain of '*N. closterium* f. *minutissima*' was the subject of investigation in the following papers: Ahmad (1930); Barker (1935); Bidwell, Krotkov & Reed (1952); Chu (1946*a*, *b*); Clarke & Mazur (1941); Dutton &

Table 1. *Comparative features of* Nitzschia closterium (*Ehr.*) *W. Sm. and* Phaeodactylum tricornutum *Bohlin*

	N. closterium	*P. tricornutum*	
		Fusiform cells	Oval cells
Length	25–100 μ. (Cupp, 1943)	Up to 25–35 μ.	8 μ.
Motility	Actively motile	Non-motile	Slowly motile
Silica wall	Two valves with irregular markings. Multiple, fraying intercalary bands (see Pl. 1, figs. 2 & 4)	None (see Pl. 1, fig. 1).	Single valve of *Cymbella type.* No girdles or intercalary bands (see Pl. 1, fig. 3)
Form of colonies	Spreading rapidly over surface in reticulate pattern with fimbriate margin	Rounded, margin entire	Spreading for short distances, margin irregular
Growth in liquid media	Cells settle and grow in mucilaginous masses	Cells separate, tend to remain suspended	Cells settle and grow in small mucilaginous clumps
Heterotrophic growth (in darkness on 0·5 %, w/v, glucose)	Yes (3 isolates tested) (Lewin, unpublished)	No (3 isolates tested)	

Manning (1941); Dutton, Manning & Duggar (1943); French (1955); Harvey (1933, 1937, 1953*a*, *b*); Hutner (1948); Jameson, Drummond & Coward (1922); Ketchum (1939*a*, *b*); Ketchum, Lillick & Redfield (1949); Ketchum & Redfield (1938, 1949); King & Davidson (1933); Leigh-Clare (1927); Levring (1945); Lovern (1936); Lucas (1936); Pace (1941); Peach & Drummond (1924); Spencer (1952, 1954); Stanbury (1931); Strain & Manning (1942); Waksman, Stokes & Butler (1937); Villeret (1955); von Brand, Rakestraw & Renn (1937, 1939); von Brand & Rakestraw (1940); ZoBell (1935). In 1954, Ryther isolated another strain of *P. tricornutum* from the Great South Bay, Long Island, N.Y., which likewise was referred to as '*N. closterium*' in the following papers: Ryther (1954, 1956*a*, *b*); Ryther & Vaccaro (1954). Riley (1943) obtained a crude culture of '*N. closterium*' from a tank containing sea water from Long Island Sound enriched with commercial fertilizer. The cells had the same dimensions as *P. tricornutum* and, as in *P. tricornutum*, dense cultures could be grown without added silicate. Unfortunately, since the culture has been lost, it is no longer possible to determine its identity. The information contained in all of the papers listed above, with the possible exception of Riley (1943), should in the future be considered as relating to *P. tricornutum*.

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EXPLANATION OF PLATE

Electron micrographs. Each scale line represents 2μ.

Fig. 1. Fusiform cells of *Phaeodactylum tricornutum* Bohlin.
Fig. 2. Silica wall of *Nitzschia closterium* (Ehr.) W. Sm.
Fig. 3. Silica walls of oval cells of *P. tricornutum*.
Fig. 4. Enlarged portion of silica wall of *N. closterium*.

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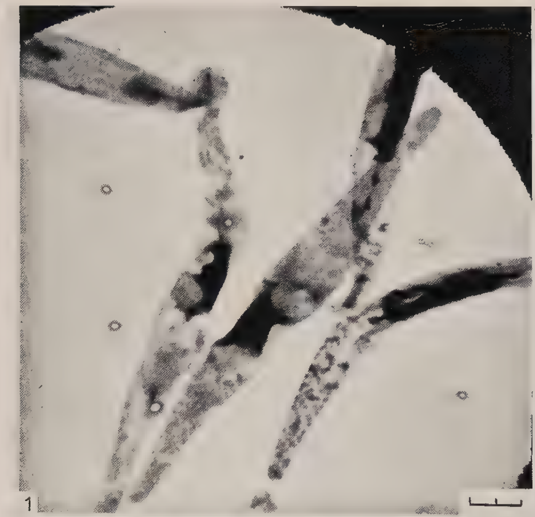
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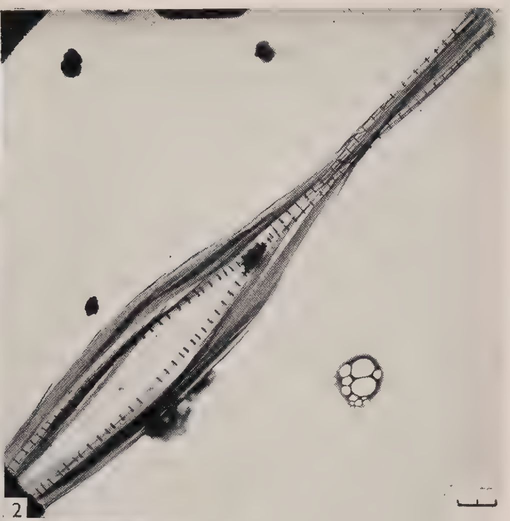
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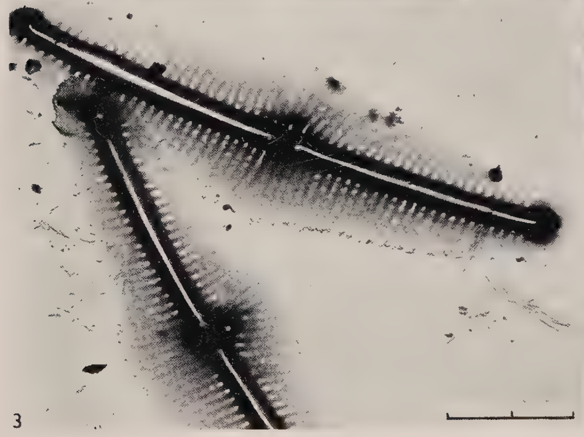
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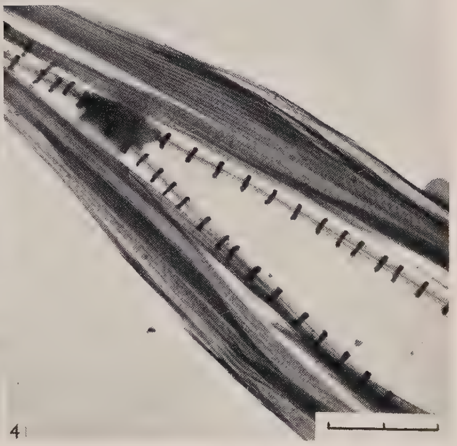
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J. C. Lewin—The taxonomy of *Phaeodactylum tricornutum*. Plate 1

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Synergistic Morphogenesis by Mixtures of *Dictyostelium discoideum* Wild-type and Aggregateless Mutants

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SUMMARY: Aggregations involving mixtures of wild-type and 'aggregateless' mutant organisms of *Dictyostelium discoideum* were examined. The proportions of wild-type individuals which could initiate the formation of aggregative centres by their neighbours was found to depend markedly upon the particular mutant in the mixture. The mutants appear to have supplied some of the cells responding to the initiative stimulus where these were needed and also to have produced diffusible material enabling many more wild-type organisms to act as initiators than would ordinarily do so. The results also indicate the existence of a variety of cellular interactions which may underlie the normal morphogenetic sequence.

Aggregation of the myxamoebae of cellular slime moulds occurs during the stationary phase. The cells elongate and move in radial streams towards centres of aggregation. Subsequently each aggregate is transformed into a slug-shaped pseudoplasmodium and ultimately into a terminal fruiting body. One experimental approach to the aggregative process was made available by the finding that myxamoebae, washed free of their bacterial associates and dispensed on washed agar, did not proliferate but could aggregate in normal fashion ultimately to produce fruits with viable spores. Some of the salient facts obtained by studies along these lines (Sussman & Noel, 1952; Sussman, 1952, 1955) follow.

(1) The number of aggregative centres produced by a population of constant size is a function of the population density. A maximum number of centres was formed at the optimal density. On the low side, progressively fewer centres appeared until at the threshold density none were formed due presumably to ineffectiveness of the chemotactic stimulus. On the high side, centres were larger but fewer, due presumably to competition between neighbouring potential centre-forming agencies. The precise values of the threshold and optimal densities varied with species and strain. (The performance of *Dictyostelium discoideum* wild-type is illustrated by the control curve in Fig. 6.)

(2) At the optimal density, the number of aggregative centres is directly proportional to the number of cells present. For *Dictyostelium discoideum* wild-type, the ratio of centres formed to cells present was 1:2200. Mutants and other species yielded different values.

(3) Not all cells in the population can initiate aggregative centres. In small replicate population samples, the ability to initiate a centre was distributed at random as a single event (i.e. statistically consistent with the Poisson series). Under these conditions the ratio of centres formed to cells present was again 1:2200.

(4) Synergistic aggregations occur when *Dictyostelium discoideum* wild-type cells, at too low a density to aggregate alone, are mixed with 'aggregateless' mutant cells, incapable of aggregating spontaneously. Small constant numbers of wild-type myxamoebae were mixed with varying numbers of aggregateless cells. The number of centres increased progressively with the addition of mutant cells until, in the presence of excess aggregateless individuals, a plateau was reached. At the plateau, the number of centres formed was directly proportional to the number of wild-type in the mixture. When mixed with one aggregateless stock, the plateau ratio of centres formed to wild-type present was 1:980; with another mutant, 1:1780.

Consideration of the first three statements compelled us to accept the view that the formation of each aggregative centre requires the presence of a special cell in the population. This cell, termed the initiator, induces its neighbours, the responder cells, to begin aggregation. The fourth statement indicated that the number of cells which can act as initiators depends upon the kind of responder cell audience which they face. The present communication, concerned with a study of synergistic aggregations by *Dictyostelium discoideum* wild-type and 25 independently isolated aggregateless strains, gives a measure of the extent to which the ratio centre:wild-type cell can vary. The results also provide an insight into the way in which this change in the distribution of centres may be effected.

METHODS

Organisms and culture methods. Dictyostelium discoideum Raper, strain NC-4, was used. Some of the mutants arose spontaneously but the majority were obtained after u.v.-irradiation by procedures previously described (Sussman & Sussman, 1953). All were isolated in pure culture by serial clonal passage, being grown upon glucose peptone agar (SM) in association with *Aerobacter aerogenes* (Sussman, 1951).

Aggregation experiments. Mass cultures on SM medium with *Aerobacter aerogenes* were incubated 40–44 hr. at 22° by which time the bacteria had been ingested and the myxamoebae were in the stationary growth phase. The myxamoebae were harvested with cold distilled water and washed three times by centrifugation for 5 min. at 1000 rev./min. in an International refrigerated centrifuge. The final pellet was suspended in salt solution (Bonner, 1947) and replicate direct counts were made in a Levy haemocytometer. Appropriate dilutions were dispensed in 0·01 ml. drops on washed agar in Petri dishes (Sussman, 1952). The Petri dishes were allowed to stand with covers ajar to permit absorption of the excess fluid by the agar and were incubated at 22°. Counts of aggregative centres were made under a dissecting microscope at the times indicated for the separate experiments.

Incubation on agar membranes. Thin agar membranes (30–60 μ. thick) were prepared on stainless-steel membrane holders by a procedure described elsewhere (Sussman & Lee, 1955) and incubated in sealed Stender dishes under a water-saturated atmosphere. Washed myxamoebae were dispensed by pipette on either or both sides. After permitting the cells to sink down on and adhere to the agar, most of the excess fluid was removed with a piece of filter paper. Incubation and counts of aggregative centres were conducted as previously described.

RESULTS

Pedigrees and special properties of the aggregateless stocks

Table 1 provides the pertinent data. As seen, three parental stocks were used: wild-type (NC-4), the bushy mutant (Bu-1), and the fruity mutant (Fty-1), themselves isolated from the wild-type after u.v.-treatment (Sussman & Sussman, 1953; Sussman, 1955).

Table 1. *Pedigree and special properties of aggregateless stocks of* Dictyostelium discoideum

Source	Strain number	Method of isolation	Special properties
Wild-type	53, 53-A, 59, 70, 71, 72, 73, 74, 78	Ultraviolet irradiation	Sharply defined plaques with no sign of aggregation when grown on SM medium with *Aerobacter aerogenes*. The myxamoebae are morphologically indistinguishable from the wild-type
	87	Spontaneous mutation	
Mutant Bu-1	204, 206	Ultraviolet irradiation	
	83, 84	Spontaneous mutation	
Mutant Fty-1	122	Ultraviolet irradiation	
Wild-type	75, 76, 77, 79, 85, 86, 89, 91	Ultraviolet irradiation	Diffuse, rapidly spreading plaques with no sign of aggregation when grown on SM medium with *A. aerogenes*. The myxamoebae are dwarfs.
Mutant Fty-1	121, 126	Ultraviolet irradiation	

The bushy mutant produces bushes of tiny fruiting bodies from each original aggregate. Washed myxamoebae on washed agar have a threshold density of 80 cells/mm.^{2} for any centre formation and an optimal density of 350; the respective values for the wild-type are 80 and 200. At the optimal density the ratio centres:cells was 1:1800, scarcely different from that of the wild-type (1:2200). The fruity mutant is distinguished by the fact that it forms a multitude of tiny aggregates and a single correspondingly small but otherwise normal fruiting body from each. The threshold density for Fty-1 was found to be 40 and the optimal density, 200. The centre:cell ratio at the optimal density is 1:24.

When grown on SM agar medium with *Aerobacter aerogenes*, all of the aggregateless mutants produced plaques (colonies) devoid of any sign of aggregation. The specific plaque morphologies did differ as indicated in Table 1. In addition, one class of mutant stocks is distinguished by the fact that the myxamoebae are dwarfs. The mean diameter of such cells is 5μ. in the stationary phase. Under the same conditions the mean diameters of wild-type or other aggregateless myxamoebae are all about 11μ., in agreement with the figure arrived at by Bonner & Frascella (1953).

Gregg (1957) showed that, of the dwarf strains, at least Agg-89 and Agg-91 differed antigenically from the wild-type. Good agglutination was obtained with homologous mixtures of myxamoebae and immune sera but no cross-reactions could be seen in heterologous mixtures. In contrast, the non-dwarf aggregateless strains tested did not differ from the wild-type antigenically by this criterion.

Synergistic aggregations

Washed suspensions of wild-type and mutant myxamoebae were prepared and counted as described in the methods section. Mixtures were made and replicate 0·01 ml. drops were dispensed on washed agar. Further treatment and counts of aggregative centres were performed as previously described.

The mutant strains fell into four classes on the basis of their performances in this experiment. These will be described separately.

Mutants Agg-53, -53-A, -70, -77, -204 (*class* 1). The mutant cells when dispensed on washed agar failed to aggregate without exception. The wild-type, in the numbers used (2500 and 5000/0·01 ml. drop), produced either no or at most about one centre/drop. Because a 0·01 ml. drop covers 30 mm.^{2} of agar surface, the wild-type myxamoebae were at densities of 83 and 166 cells/mm.^{2} respectively. The former is at the threshold for any centre formation at all. The latter permits only about 50 % of the number of centres which normally would form at the optimal density of 200 cells/mm.^{2} (Sussman & Noel, 1952). In the case of 5000 wild-type cells this would be 50 % of 2·3 centres.

The mixtures of wild-type and mutant behaved as follows. The addition of aggregateless cells permitted a progressive increase in the number of centres until a plateau was attained. The plateau was conserved even with as many as 6×10^5 mutant cells/drop, a density of about 2×10^4 cells/mm.^{2}. Moreover, at the plateau, the number of centres was proportional to the number of wild-type present. Representative curves are shown in Figs. 1 and 2. Data relating to the constancy of the ratio centre: wild-type cell at the plateau are given in Table 2 for three of the mutants, and for the remaining two in Table 5.

Mutants Agg-85, -89, -91 (*class* 2). The synergistic performance of these strains is illustrated by the data given in Fig. 3. The numerical values for the ratio centre: wild-type cell at the plateau for Agg-91 are shown in Table 3. It may be seen that where small numbers of wild-type cells were used (500–1000), the curves were equivalent to those obtained with the mutants in class 1 above. That is, the number of centres formed at the plateau was proportional to the number of wild-type cells present and thus the ratio plateau content: wild-type cell remained constant at about 1:118. However, where higher numbers were used (2500–8000) the proportionality was not retained and the ratios progressively decreased. To account for this alteration, it must be noted that an increase in wild-type cells from 500 to 8000 within a drop of constant volume (0·01 ml.) involves a 16-fold increase in population density of the wild-type. An experiment was accordingly designed in which constant numbers of wild-type could be spread over a range of densities and yet still be in the presence of excess mutant cells. Replicate 0·01, 0·03, and 0·05 ml. drops were dispensed on washed agar. (The mean areas covered by these drops were 30, 66, and 105 mm.^{2}, respectively.) Each drop contained 8000 wild-type but the number of aggregateless cells varied with the size of the drop so as to provide a

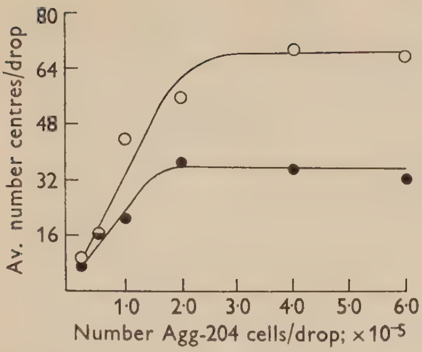

Fig. 1

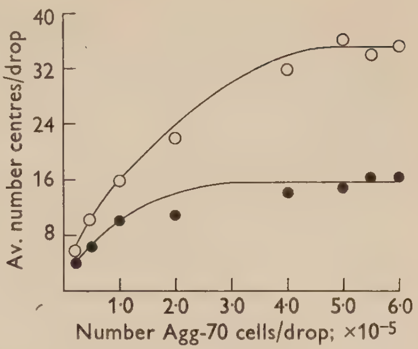

Fig. 2

Fig. 1. Mixtures of *Dictyostelium discoideum* wild-type and Agg-204. ○ = 5000 wild-type cells/drop; ● = 2500 wild-type cells/drop. Each point on the curves represents at least 32 separate determinations. Counts were made at 24 and 48 hr.

Fig. 2. Mixtures of *Dictyostelium discoideum* wild-type and Agg-70. ○ = 5000 wild-type cells/drop; ● = 2500 wild-type cells/drop. Each point on the curves represents at least 50 separate determinations. Counts were made at 24 and 48 hr.

Table 2. *The ratios of centres formed to wild-type cells present when the latter were mixed with 3 aggregateless mutants of class* 1

Aggregateless strain employed	Number of wild-type present	Mean number of centres formed at plateau	Ratio of centres : wild-type cells present
Mutant Agg-70	5000	35	1:143
	2500	16·3	1:153
Mutant Agg-204	5000	67·8	1:74
	2500	32·2	1:78
Mutant Agg-77	5000	12	1:417
	2500	6·7	1:373

Table 3. *Ratios of centres to wild-type cells in mixtures of the latter with mutant Agg*-91

Number of wild-type in mixture	Centre : wild-type cell ratio at plateau
500	1:107
750	1:129
1000	1:118
2500	1:230
5000	1:310
8000	1:442

constant density of 2×10^4 mutant cells/mm.^{2}, well above the value required to insure a plateau value for centre formation. Similar series of drops were dispensed containing 5000 and 2500 wild-type. The formation of centres by these mixtures is summarized in Table 4.

In the presence of excess aggregateless cells, 2500 wild-type cells at a density of 83 wild-type/mm.^{2} induced the formation of 10·9 centres. The same number at a density of 38 wild-type/mm.^{2} induced 13; at a density of

Table 4. *The dependence of centre formation on wild-type population density in synergistic mixtures of wild-type and mutant Agg*-91

Number of wild-type cells present		Drop volumes (ml.) 0·01	0·03	0·05
8000	Wild-type population density (cells/mm.^{2})	263	121	76
	Number of centres	18·2	22·5	38
	Ratio centres: wild-type cells	1:442	1:326	1:210
5000	Wild-type population density	166	76	48
	Number of centres	16·1	19	27
	Ratio	1:310	1:260	1:185
2500	Wild-type population density	83	38	24
	Number of centres	10·9	13	18·6
	Ratio	1:230	1:192	1:134

24 wild-type/mm.^{2}, 18·6 centres. It is therefore clear that by decreasing the wild-type density more centres were induced. In fact, at the lowest density, the ratio centre: wild-type cell was 1:134, bringing it within range of the ratios attained with 500–1000 wild-type, and thereby extending the proportionality between centres and wild-type cells. The data for 5000 and 8000 wild-type are consistent with this conclusion, although the plateau ratios still had not reached the value 1:118 at the lowest wild-type densities employed.

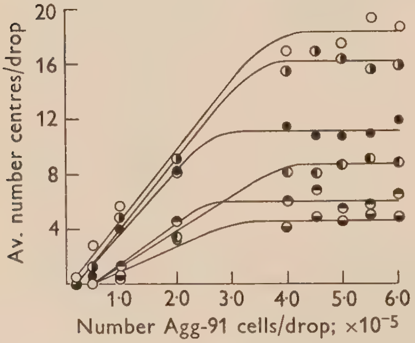

Fig. 3

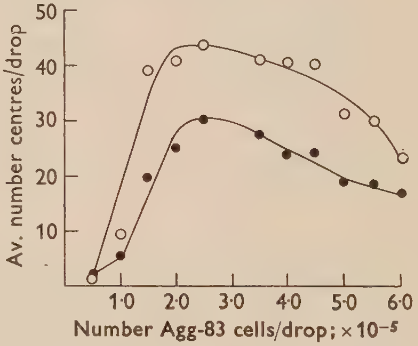

Fig. 4

Fig. 3. Mixtures of *Dictyostelium discoideum* wild-type and Agg-91. ○=8000 wild-type cells/drop; ◑=5000; ●=2500; ◐=1000; ◓=750; ◒=500. Each point on the curve represents at least 50 separate determinations. Counts were made at 24 and 48 hr.

Fig. 4. Mixtures of *Dictyostelium discoideum* wild-type and Agg-83 showing an inhibition of aggregative centre formation at high concentrations of Agg-83. ○=5000 wild-type cells/drop; ●=2500 wild-type cells/drop. Each point on the curve represents at least 30 separate determinations.

The situation here encountered is seen to be somewhat analogous to that which obtains when wild-type myxamoebae serve as sole responders to their own initiators. In the latter circumstances, fewer centres are formed at densities above optimal (200 cells/mm.^{2}) than at it. The difference between the two systems lies in the fact that though this interference also operates when small numbers of wild-type are faced with a saturation audience of mutant

Agg-91, it does so at densities as low as 38 cells/mm.². Clearly then, the presence of the mutant is required for the interference to operate at so low a density and it is fair to assume that the mutant exerts an interactive effect upon the wild-type to this end. But it must be recognized that this interference ultimately involves an interaction among the wild-type themselves since the diminution in centre formation becomes evident when the wild-type are brought closer to each other while yet maintaining a constant density of mutants.

Cellular interactions which mediate and control differentiation are commonly encountered in development systems (Grobstein, 1956) and in fact represent one of the problems basic to developmental biology. The above type of interaction is particularly interesting in that it involves the interaction of one cell class upon another which in turn causes the cells of class 2 to interact amongst themselves. The noteworthiness of these considerations stems from the fact that not even the ground rules of interactive relationships during development are known as yet, much less their biochemical mechanisms.

Mutants Agg-59, -71, -72, -73, -74, -78, -83, -84, -87, -206 (*class* 3). The synergistic capabilities of these mutants are complicated by the fact that when present in great numbers they inhibit centre formation. In some cases, the degree of inhibition is fairly slight as is illustrated in the results obtained by mixing wild-type with Agg-83 (Fig. 4). Here, centre formation rose to a maximum rather than a plateau and sharply declined with increasing numbers of mutant cells. Mixtures with Agg-74 and -206 gave similar results. In other cases, the degree of inhibition was more severe and the ability to form centres disappeared completely when the number of mutant cells exceeded a range of 5×10^4 to 2×10^5, depending on the particular strain used (Agg-59, -71, -73, -78, -84). At the maxima, the numbers of centres were generally not proportional to the number of wild-type present but rather showed a steadily decreased slope. However, in a few strains proportionality was conserved. Two extreme cases of inhibition were observed (Agg-72, -87). No centres appeared in the mixtures no matter what the concentration of mutant cells. In fact, these mutants prevented centre formation even when the wild-type was present at a density high enough for it to aggregate alone.

Mutant Agg-121. A most remarkable performance was observed in mixtures of wild-type and this mutant; the data are summarized in Fig. 5. First, a double plateau was encountered. Attempts to show that this was due to the fact that the Agg-121 population contained two kinds of cells reacting differently to the presence of wild-type were uniformly unsuccessful. Secondly, the number of centres depended hardly at all upon the number of wild-type present within the range of 2500–5000 cells. This anomalous system is being further studied.

The spectrum of centre: wild-type cell ratios

Table 5 summarizes the best estimates of the ratio centre: wild-type cells. The figures without parentheses are firm estimates, based upon proportionate series of two or more plateau or maxima. The figures within parentheses were arrived at from non-proportionate series and represent the highest ratios observed in each series. The data further fortify the argument that the number of cells in the wild-type population which can act as initiators depends to a marked extent upon the nature of the audience which must be excited. As shown in Table 5 the most sensitive audience was mutant Agg-204, since they could be induced to form centres by one out of 76 wild-type cells. However, it is clear that the response of any mutant population can not be taken simply as a reflexion of the efficiency of its sensing apparatus. Thus the performances of mutants Agg-72 and -87, were complicated by the inhibitory effect which they exerted upon the wild-type; in these cases no assessment of sensitivity is possible.

Table 5. *The spectrum of centre:wild-type cell ratios permitted by the aggregateless mutants*

See text for detailed explanations.

Class*	Mutant	Centre:wild-type cell ratio
1	53	1:980
	53-A	1:1780
	70	1:148
	77	1:395
	204	1:76
2	85	(1:300)
	89	1:210
	91	1:118
3	74	1:430
	83	(1:84)
	206	(1:298)
	59	(1:500)
	71	(1:1020)
	73	(1:940)
	78	(1:675)
	84	(1:135)
	72	(1:∝)
	87	(1:∝)
4	121	(1:360)

* See p. 436.

The synergistic relationship: the effect of wild-type upon mutants

The first question examined was: can the mutants aggregate under conditions where they are in close physiological contact with the wild-type but are physically separated? The point was tested by incubating washed cell suspensions on opposite sides of thin agar membranes. Agar membranes had been used (Sussman, 1954) in a study of synergistic fruiting by paired combinations of 13 morphogenetically deficient mutants including 6 aggregateless strains (five of which have been used in the present study) and 7 fruitless mutants which could aggregate but develop no further (i.e. unable to produce a pseudoplasmodium and terminal fruiting structure). When mixed in the proper cell ratios many paired combinations of these strains aggregated and produced terminal fruiting bodies but when incubated on opposite sides of agar membranes, they did not do so. Instead, they developed only to the extent that they did when alone. Further, when wild-type cells were opposed either by additional wild-type or by fruitless mutant cells across a membrane, the two populations aggregated in precise coincidence, each centre and radial cell stream above the membrane being reproduced in detail on the underside.

Wild-type populations eventually produced pseudoplasmodia and subsequently terminal fruiting bodies while the fruitless populations stopped development after aggregating just as they normally do when alone. In contrast to the above, the aggregateless strains simply clustered immediately below wild-type centres and streams but remained unelongated and loosely packed. Pl. 1, fig. 1 shows a photomicrograph of this response. When the wild-type streams moved into their respective aggregates the 'pseudo-streams' remained in their original positions, the individual cells staying at the sites to which they had first been attracted. Only a few of the aggregateless strains had been tested in this fashion.

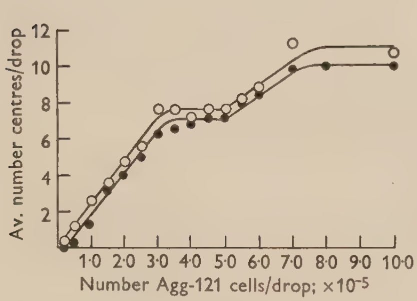

Fig. 5

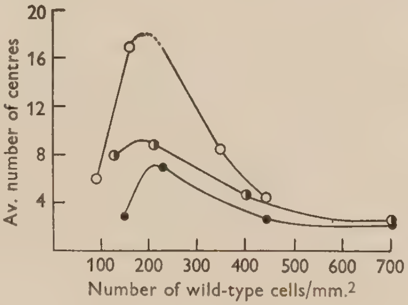

Fig. 6

Fig. 5. Mixtures of *Dictyostelium discoideum* wild-type and Agg-121, showing the double plateau. ○ = 5000 wild-type cells/drop; ● = 2500 wild-type cells/drop. Each point on the curve represents at least 100 separate determinations.

Fig. 6. Population density experiment, with 20,000 wild-type cells/drop, performed on a washed-agar substrate with the addition of an extract of mutant Agg-70 cells. ○ = 240 ml. washed-agar + 60 ml. undiluted extract; ◑ = 240 ml. washed-agar + 60 ml. 1/25 dilution of extract; ● = 240 ml. washed-agar + 60 ml. distilled water. Each point on the curve represents at least 20 separate determinations. Counts were made at 24 and 36 hr.

Since then the entire collection of 25 mutant stocks has been surveyed. No aggregateless mutant could produce a normal, complete aggregate when incubated across a membrane from the wild-type. In most cases they reacted precisely as described above. In a few cases, they remained completely unaffected and did not even cluster in coincidence with wild-type centres and streams. It would appear therefore that while at least some of the aggregateless cells can be chemotactically attracted at a distance by the wild-type, a separation of as little as 30 μ. (less than 3 cell diameters) can prevent the consummation of the synergistic relationship. Considering the present state of ignorance regarding the biochemical events which accompany aggregation and in particular the chemotactic process, it would be premature to offer a rationale for these observations.

The second question concerns the relative extents to which the mutant stocks did respond at a distance from the wild-type. One may ask, is there a correlation between the intensity with which a mutant reacts synergistically with wild-type in cell mixtures and the sensitivity with which it responds to the chemotactic apparatus of the wild-type when separated by a membrane? The relative responses of the 25 aggregateless stocks at a distance from the wild-type fell into three arbitrary classes, they were:

(*a*) Mutant cells did not respond at all to juxtaposed wild-type centres and streams but remained randomly distributed over the agar surface (mutants Agg-75, -76, -77, -79, -85, -86, -89, -91, -121, -126).

(*b*) The mutant cells clustered directly below wild-type centres but did not concentrate at the streams (mutants Agg-53, -59, -74, -83, -204, -206).

(*c*) The mutant cells clustered directly below wild-type centres and streams and more or less precisely duplicated the branchings of the latter. After the wild-type had completed its aggregation, the superimposed mutant streams remained as such or else were broken up and the cells were redistributed. The mutant cells did not elongate or pack closely even in the strongest reaction observed (mutants Agg-53-A, -70, -71, -72, -73, -78, -84, -87, -112).

Strains like mutant Agg-72 which formed no centres at all when mixed with wild-type were attracted very strongly across the 30μ. of agar membrane. In contrast, the dwarf strains like mutants Agg-77 and -89 which readily formed centres when mixed with wild-type did not react at all when separated from them. Finally, whereas only one out of 1780 wild-type would induce Agg-53A cells to aggregate when mixed, one out of 76 wild-type were able to do so when mixed with Agg-204. Yet Agg-53A reacted more intensely than Agg-204 to the wild-type when separated by a membrane. Thus it is again obvious that the total synergistic performances of the mutant stocks can not be explained consistently in terms of a single parameter, in this case the chemotactic apparatus.

The synergistic relationship: effect of mutants upon wild-type

The data given here and previously (Sussman, 1952) reveal that the aggregateless mutants can affect centre formation in mixtures with wild-type under two entirely different circumstances. The first of these involves mixtures which contain relatively small numbers of wild-type (500–5000), too sparse by themselves to form any centres at all or, at most, too far below the optimal density to form more than a fraction of the centres of which they are otherwise capable. The addition of mutant cells not only permitted centres to form but led to the appearance of many more aggregates than the wild-type population itself could have produced at any density. The second circumstance involves mixtures containing relatively large numbers of wild-type (6000–10,000) which by themselves would be at a density equal to or greater than optimal and so could form aggregates perfectly well without assistance from added mutant cells. Yet the addition of mutants led again to the appearance of many more centres than the wild-type could alone have produced. Further, when at least two of the mutants (Agg-53 and -53A) were present in excess, the ratios of centres: wild-type cells remained constant regardless of whether small or large numbers of wild-type took part in the synergistic aggregation (Sussman, 1952).

The mutants' ability to exert these two kinds of effect when physically separated from the wild-type was examined by paired incubation on thin agar membranes. A 0·01 ml. drop containing either 2000 wild-type (density = 67 cells/mm.^{2}) or 20,000 (density = 670 cells/mm.^{2}) was dispensed on one side of a washed-agar membrane and, after the excess fluid had been absorbed, a second drop of volume 0·03 ml. containing 3×10^5 mutant cells was dispensed on the other side. Control membranes bearing wild-type cells only were also prepared. Counts of aggregative centres produced by the wild-type were made at 24 hr. The drops containing 2000 wild-type produced no centres whatever, whether in the presence or absence of mutant cells on the other side of each membrane. Thus, under conditions where the wild-type is too sparse to aggregate alone, the mutant cells must actively take part if aggregation is to occur and the supply of diffusible materials is not sufficient for this purpose.

Table 6. *The ability of aggregateless cells to stimulate centre formation by wild-type when separated by an agar membrane*

See text for experimental details.

Mutant	Ratio of centres : wild-type cells observed in mixtures	Number of centres formed by 20,000 wild-type cells on agar membranes: Experimental	Control	Fold increase
Agg-204	1:76	34·2	3·6	9·5
Agg-70 (3×10^5 cells/drop)	1:148	30·1	3·5	8·6
Agg-70 ($1{\cdot}5 \times 10^5$ cells/drop)	1:148	19·1	3·5	5·4
Agg-53	1:980	5·3	3·2	1·7
Agg-72	1:∞	13·8	—	3·8

The drops containing 20,000 wild-type gave the quite different results shown in Table 6. Three noteworthy facts are apparent. First, under conditions where the wild-type could aggregate unassisted, the passage of diffusible material from the mutants permitted the wild-type itself to form a multiplicity of aggregates. Secondly, the intensity of this effect depended on the number of mutant cells present, at least in the case of mutant Agg-70. Thirdly, the intensity of the effect was strain specific. For mutants Agg-53, -70, and -204, the capacity to enhance centre formation at a distance stood in the same relation as their respective abilities to do so when mixed with wild-type. Mutant Agg-72 is anomalous in that, while it actually inhibited centre formation when mixed with wild-type, it stimulated centre formation at a distance from the latter.

To summarize, the foregoing indicates that the aggregateless cells perform at least two functions in the synergistic mixtures: (*a*) they take an active part in the aggregation as responder cells and can offset the lack of wild-type responders in doing so. This function is necessary only where a lack of responder cells exists; (*b*) they produce diffusible material affecting the wild-type cells in such a way as to lead to a multiplicity of centres. When the number of wild-type in the mixture is small, both functions are performed by the mutants. When the number of wild-type is large, only the latter function (*b*) is necessary to produce the synergistic result.

The effect of cell-free extracts on centre formation by the wild-type

The results of the last described membrane experiments raised the question of whether cell-free preparations from mutant stocks might not be able to affect wild-type aggregation in similar fashion. Preliminary results indicate that they do and further, that active preparations can be obtained not only from aggregateless cells but also from the fruity mutant Fty-1, and from the wild-type itself. To prepare extracts, the cells were harvested at the beginning of the stationary phase and ground in a mortar with powdered dry ice. The crude brei was cleared by centrifugation and taken to pH 3·5, whereupon a heavy proteinaceous precipitate settled out. The precipitate (inactive upon assay) was discarded; the supernatant fluid was neutralized and mixed with a suitable amount of molten washed agar and plates poured therefrom. Streptomycin sulphate (0·2 %, w/v) was included to prevent growth of bacteria and, in consequence, of the myxamoebae (Sussman, 1955).

Table 7. *The effect of cell-free extracts on centre formation by* Dictyostelium discoideum *wild-type*

Source of extract	Activity*
Mutant Agg-70	2·6
Mutant Agg-72	2·5
Mutant Fty-1	11·4
Wild-type	7·8

* Ratio of the number of centres produced by 20,000 wild-type cells on extract-agar to number produced on washed-agar.

Bioassays were performed as follows. Appropriate dilutions of washed wild-type cells in salt solution were dispensed in drops on extract and control agars. The drops contained 20,000 cells over a range of population densities. The densities were determined by direct count with an ocular grid and were also calculated from measurements of the areas covered by the drops. Fig. 6 shows one assay of an extract obtained from mutant Agg-70. The extract did not affect the threshold or optimal density values nor did it alter the depression in number of centres encountered at densities above optimal. However, significantly greater number of centres appeared. At the optimal density the increase over the control value was 2·6-fold. Figure 7 shows the assay of a much more potent preparation from mutant Fty-1. An 11·4-fold increase in number of centres occurred at the optimal density. Table 7 contains a summary of the best results obtained with extracts from four strains of *Dictyostelium discoideum.* It is impossible to compare the relative potencies of these strains because replicate preparations have shown great fluctuation in activity. For one thing, the age of the culture at harvesting is extremely critical. Log phase cells provided inactive extracts. Cells considerably past the beginning of the stationary phase were much less active. In addition, the activity proved to be temperature-sensitive and degenerated upon storage, even in the cold. Notwithstanding these difficulties it may be said that preparations from mutant Fty-1 and wild-type were generally more active than those from the aggregateless strains although overlap was encountered. Previous studies (Bradley, Sussman & Ennis, 1956; Sussman, 1956) showed that the initiative capacity of the wild-type is immune to a wide variety of external influences both chemical and physical. Thus the effects described above are at present unique and therefore represent a promising opportunity to examine the biochemical mechanisms underlying the initiation of aggregative centres.

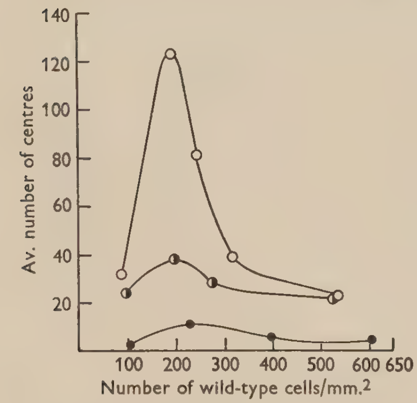

Fig. 7

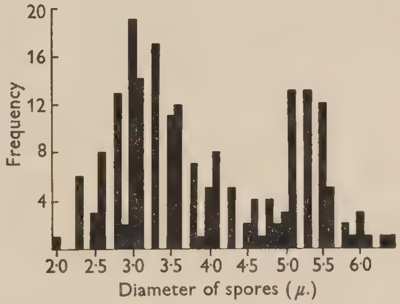

Fig. 8

Fig. 7. Population density experiment, with 20,000 wild-type cells/drop, performed on a washed-agar substrate with the addition of an extract of mutant Fty-1 cells. ○ = 240 ml. washed-agar + 60 ml. extract. ◑ = 240 ml. washed-agar + 60 ml. 1/5 dilution of extract. ● = 240 ml. washed-agar + 60 ml. water. Each point on the curve represents 4 separate determinations.

Fig. 8. Size distribution of spores from fruiting bodies obtained by mixing 500 wild-type cells with 6×10^5 Agg-91 cells in a 0·01 ml. drop on a washed-agar medium. The size is given as the mean of the major and minor diameters. Two hundred spores were measured.

The entrance of mutant cells into synergistic aggregates

That cells of at least some aggregateless strains actually do enter the synergistic aggregates has been demonstrated in two ways. Spores from the resulting fruiting bodies were plated in clonal isolation to yield appreciable proportions of aggregateless clones, the precise values depending upon the nature of the paired strains and the proportions of myxamoebae originally mixed (Sussman, 1954). R. R. Sussman (unpublished data) vitally stained aggregateless cells and mixed them with wild-type. She found the stained cells in all parts of the fruiting structures.

The experiments to be described involved mixtures of wild-type and mutant Agg-91, and the results indicated that Agg-91 entered the aggregate and gave rise to dwarf spores which were incapable of germinating under conditions which permit wild-type spores to do so. Drops containing 500 wild-type and 6×10^5 cells of mutant Agg-91 were dispensed on washed-agar and allowed to fruit. Spore masses were suspended in water and examined in wet mounts. Two kinds of spores were evident: those resembling wild-type, and dwarf spores. Pl. 2 illustrates the size differences between these spores and between the corresponding vegetative cells. Measurements were made of the major and minor diameters of a few hundred individuals in the mixed spore population. The histogram Fig. 8 shows a bimodal distribution, one set of spores with a mean diameter of about $5{\cdot}4\mu$. and the other set, about $3{\cdot}0\mu$; the former value is characteristic of wild-type spores (Bonner & Frascella, 1953).

Table 8. *Efficiency of plating* and % germination of spores from mixtures of* Dictyostelium discoideum *wild-type and Agg*-91

Number of wild-cells per drop	Number of spores plated	Plaque count	EOP*	Initial spore count	Number of non-germinated spores after 24 hr.	% germination of spores
5000	—	—	—	2008	188	90·6
1000	1595	588	0·37	3476	1341	61·4
	4149	1923	0·46			
500	1575	290	0·18	—	—	—
NC-4 alone	1591	1290	0·81	1194	26	97·8

* Plating efficiency = plaque count/no. spores plated.

Spores samples obtained from mixtures of 6×10^5 cells of mutant Agg-91 and different numbers of wild-type were plated out on SM agar with *Aerobacter aerogenes*. Among thousands of clones which appeared, only wild-type was encountered. However, the plating efficiency was positively correlated with the relative proportion of wild-type myxamoebae in the mixture (Table 8). The spores were also dispensed on SM agar without bacteria to determine % germination, the same correlation was encountered. In addition, the ungerminated spores were almost exclusively of the dwarf type.

The conclusion therefore appears inescapable that Agg-91 cells gave rise to the non-viable dwarf spores. That being so, the efficiency of plating and % germination values would seem to be reliable measures of the number of mutant cells which enter a synergistic aggregate (the spores make up 85 % of the cellular assembly in a fruit, Bonner & Slifkin, 1949; Sussman, 1955). Table 8 indicates that the mutant cells were a progressively decreasing part of the aggregates as the number of wild-type in the mixture increased. In drops containing more than 5000 wild-type the cellular contribution of the mutant would appear to be negligible. This is consistent with the conclusion derived from the agar membrane studies, namely that, where the number of wild-type is sufficient to permit them to aggregate alone, the synergistic contributions of the mutants need only to be in the form of diffusible material.

Mixtures of aggregateless strains

The elegant mutational analyses of *Neurospora crassa, Escherichia coli*, and other organisms have shown that simple metabolic lesions can account for the inability of trophically deficient mutants to grow under prescribed conditions. It seemed reasonable therefore to suppose that simple blockages of biosynthetic pathways might account for the inability of at least some morphogenetically deficient mutants of *Dictyostelium discoideum* to develop normally under defined conditions. With *E. coli* mutants Davis (1950) showed that strains possessing different lesions could mutually satisfy their deficiencies and thus grow synergistically on minimal medium. One might equally well expect that two different aggregateless stocks of *D. discoideum* would in the same way be capable of synergistic aggregation. A previous study tested this possibility with paired and trebled combinations of six independently derived aggregateless mutants which had shown clear phenotypic differences (Sussman, 1954); no synergistic aggregations were then observed. This survey has now been extended to 25 mutants. Of the 300 possible pairings, 217 were examined on washed-agar without success. Three different numbers of cells of each strain were mixed in all permutations. In addition, 79 pairs were grown together on SM agar with *Aerobacter aerogenes.* These produced no aggregates; neither did mixtures on washed-agar in which the positions of the partners were assymetrical It should be noted that syntrophy between *E. coli* mutants as described by Davis requires that there be accumulation of intermediates which occur before the metabolic lesion and that these be excreted in appreciable amounts. It is possible that these requirements are not met by the aggregateless strains of *D. discoideum* used here. On the other hand perhaps morphogenetic assemblies require for their proper functioning a delicately balanced matrix of interactions correctly oriented in space and time and that this is impossible to duplicate simply by mixing two unbalanced mutants.

DISCUSSION

The synergistic performance of *Dictyostelium discoideum* wild-type paired with eight of the aggregateless strains has provided evidence supporting the thesis that single wild-type cells are specially endowed to initiate aggregations: in the presence of excess mutant cells of these eight varieties (Agg-53, -53A, -70, -74, -77, -89, -91, -204), the number of centres formed was linearly proportional to the number of wild-type present. If two or more wild-type cells acted in concert to induce centre formation the relationship would have been exponential, not linear.

The ten other aggregateless strains which were examined gave equivocal results. That is, the number of centres was not linearly proportional to the number of wild-type with which these mutants were paired. However, the centre: wild-type cell relationships showed a decreasing slope with increasing numbers of wild-type—the reverse of an exponential function. Secondly, the results obtained with mixtures of wild-type and mutants Agg-89 or -91 have shown that the linear relationship may hold only within a limited range of wild-type population sizes and be obscured outside of that range because of extraneous circumstances. Perhaps, therefore, a more detailed examination of the synergistic activities of the ten strains will reveal the existence of such limited ranges of proportionality.

There is now a considerable body of evidence to suggest that the number of cells which can act as initiators depends markedly upon the nature of the audience which must be induced to aggregate. For the wild-type the proportion of initiators has been found to be either 1:2200 or 1:76, depending upon whether the audience consists of wild-type themselves at relatively low density (200 cells/mm.^{2}) or of mutant Agg-204 at very high density (2×10^4 cells/mm.^{2}). For the fruity mutant Fty-1, the ratio is either 1:24 or 1:63, depending on whether the fruity cells supply their own audience or whether the audience is made up of wild-type (Sussman, 1955).

At least two explanations may be invoked to account for these variations in initiator cell ratios, namely:

(1) There is a random distribution of initiative capacity in the population. A small proportion of cells possess high capacity; the remainder possess moderate or low capacities. In any system the number of cells which are potentially strong enough initiators actually to induce aggregation is determined by the innate sensitivity of the responder cells as well as by how closely the latter can be packed around the putative initators so as to receive the aggregative stimulus.

(2) The matrix of interactions within a population before the onset of aggregation can induce cells to become initiators; these then supply the aggregative stimulus. The higher the density at which the cells can be packed, the more intense can these interactions be and thus the greater the number of initiator cells. Wild-type cells can be packed only loosely because at densities above 200 cells/mm.^{2} the initiator cells are already so close that they compete for the same audience and thus cannot make their presence known. But in synergistic mixtures, the wild-type is maintained at constant low density and it is only the aggregateless cells which are packed closely, thus permitting more intense interactions and the concommitant production of many more demonstrable initiators amongst the sparsely packed wild-type.

This second explanation has been given support by the experiments involving extracts of mutant and wild-type strains. However, they are not mutually exclusive and both may operate.

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Part of the experimental results reported here were taken from a thesis offered by the senior author (H. L. E.) in partial fulfilment of the requirements for the Ph.D. degree at Northwestern University.

The senior author was an N.S.F. pre-Doctoral Fellow during the course of this investigation. He is currently a post-Doctoral Fellow, National Institutes of Health, U.S. Public Health Service, at Northwestern University.

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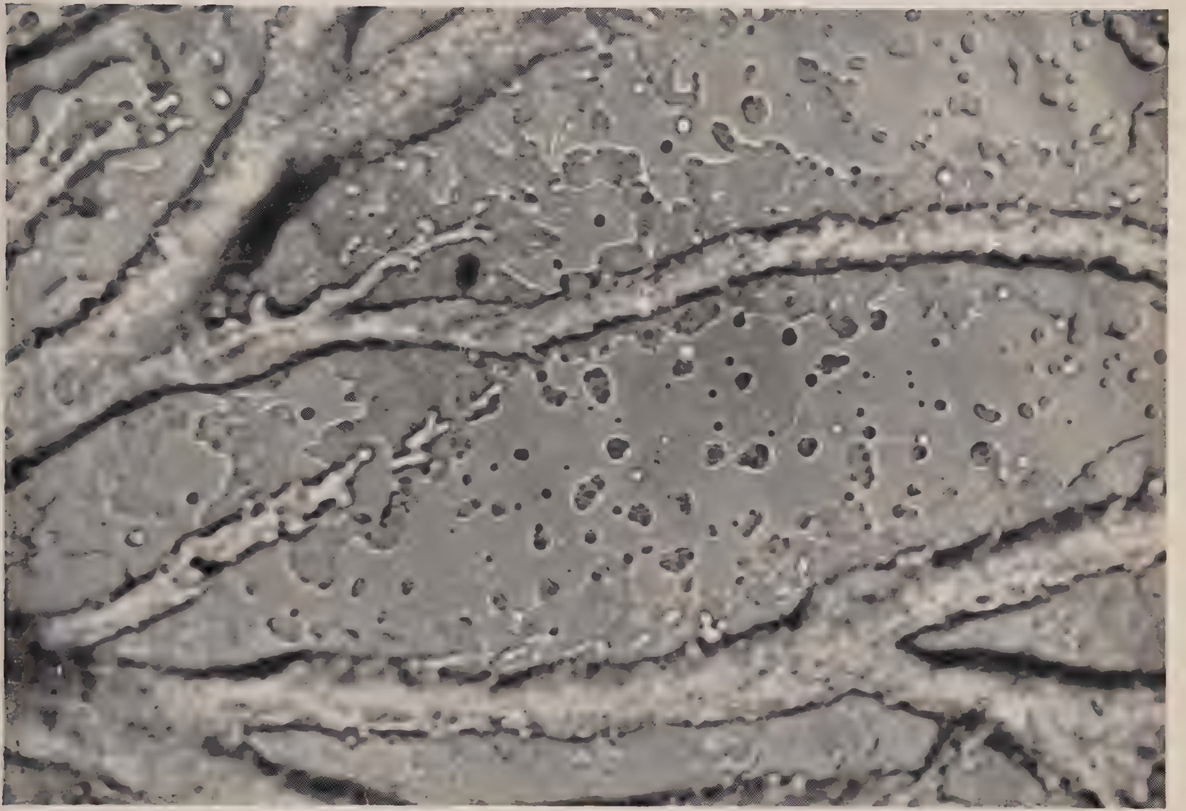

Fig. 1

H. L. Ennis and M. Sussman—Synergistic morphogenesis. Plate 1

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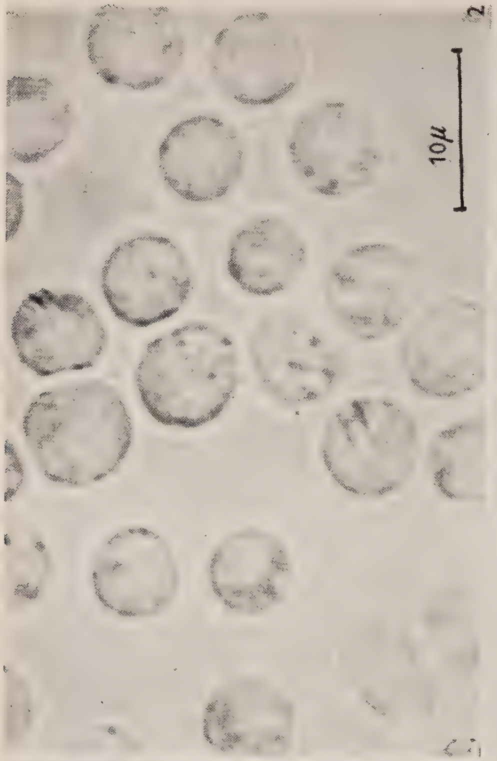

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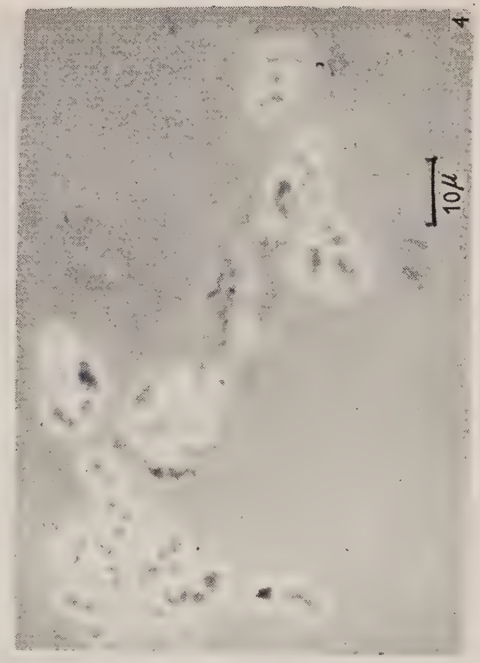

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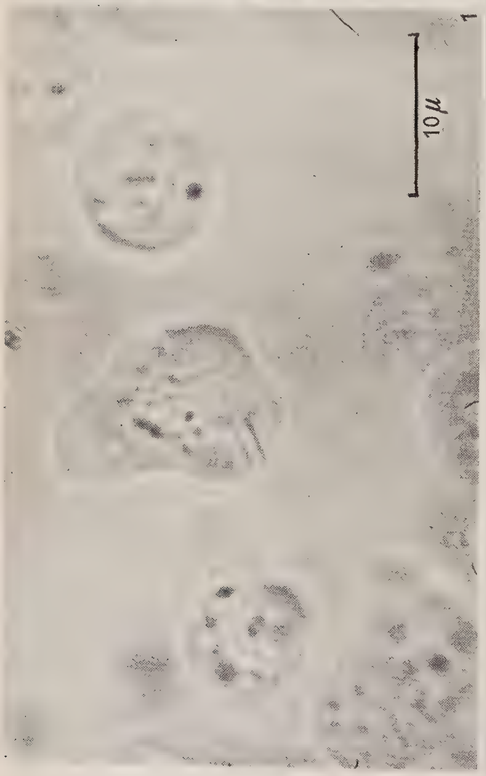

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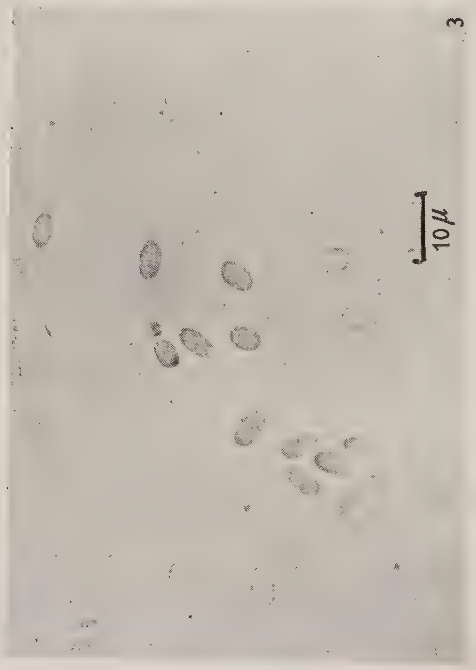

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H. L. ENNIS AND M. SUSSMAN—SYNERGISTIC MORPHOGENESIS. PLATE 2

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EXPLANATION OF PLATES

PLATE 1

1. Attraction of *Dictyostelium discoideum* mutant Agg-78 to wild-type aggregative streams across a thin agar membrane. The wild-type stream is well formed and tightly packed. The Agg-78 cells may be seen to cluster around these streams but do not produce well-formed streams of their own. Magnification, × 860.

PLATE 2

1. *Dictyostelium discoideum* wild-type vegetative myxamoebae.
2. Mutant Agg-91 vegetative dwarf myxamoebae. Note difference in size between the two strains.
3. Spores from wild-type fruiting bodies. Note the small variation in spore sizes in the sample.
4. Spores from fruiting bodies formed from a mixture of 500 wild-type and 6×10^5 Agg-91 cells plated in 0·01 ml. drops on washed agar. Note the great variation in size among the spores and the preponderance of small spores, presumably derived from Agg-91 dwarf myxamoebae.

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Relationship between Beet Ringspot, Potato Bouquet and Tomato Black Ring Viruses

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SUMMARY: Serological and plant-protection experiments provided evidence that potato bouquet and beet ringspot viruses are strains of tomato black ring virus. Tomato black ring and potato bouquet viruses are more closely related to each other than either is to beet ringspot virus. The three produce similar symptoms and have a similar host range. There was no evidence that they are related to tobacco ring spot or peach yellow bud mosaic viruses. In the plant-protection tests, protection between serologically related virus strains often was not reciprocal; when protection was incomplete, the symptoms produced by the second virus were milder than those in plants infected by it alone. Strains of tomato black ring virus could be arranged in order of their ability to cause symptoms in plants already infected with other strains. Although results of the plant-protection and serological tests agreed broadly, the correlation was not complete, suggesting that the tests measure different properties of the virus particles. The degree of difference between strains of tomato black ring virus seems to reflect their geographical separation, something that is perhaps to be more expected with soil-borne than with other viruses.

Several distinct plant viruses cause similar symptoms of the ringspot type in tobacco and other host plants, so that symtomatology is not a reliable guide to identity. Two kinds of tests group such virus isolates as related strains or distinguish them as different viruses. First, related virus strains react with one another's antisera, and secondly, infection with one strain protects plants from infection with another. Although there are examples of serologically related viruses which do not protect plants from one another, and of plants infected with one virus which are refractory to a second apparently unrelated one (Bawden & Kassanis, 1945, 1951), the plant-protection test has usually worked well with ringspot viruses (Tall, Price & Wertman, 1949).

It was mainly similarity of symptoms which led Köhler (1950) to suppose that a virus isolated in Germany from potatoes with bouquet disease was a strain of the North American tobacco ring spot virus, but the serological and plant-protection tests done by Bercks & Gehring (1956) show little or no relationship between the two. Potato bouquet is very like the potato disease caused by the soil-borne beet ringspot virus found in Scotland (Harrison, 1957*a*). Beet ringspot virus also infects plants of many other species (Harrison, 1957*a*, *b*), and the symptoms produced in peach resemble those caused by peach yellow bud mosaic virus which is soil-borne in California (Thomas, Scott, Wilson & Freitag, 1944; Wagnon & Breece, 1955); in some other species the symptoms caused by beet ringspot virus resemble those produced by tomato black ring virus, described in England (Smith, 1946). Because tobacco plants infected with beet ringspot virus were not immune from potato bouquet virus and because beet ringspot virus antiserum when diluted 1/35 precipitated beet ringspot but not potato bouquet virus, these two were considered distinct (Harrison, 1957*a*). However, further experiments showed that tobacco plants infected with potato bouquet virus were immune from beet ringspot virus, and suggested that a more detailed study of the relationships between the viruses mentioned above would be worthwhile. The results are reported in this paper.

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METHODS

All the viruses were propagated in tobacco plants (*Nicotiana tabacum* L. var. White Burley). The two isolates of beet ringspot virus usually used were originally isolated from potato and burdock (*Arctium lappa* L.); no differences between them were detected in the plant-protection and serological tests. Potato bouquet virus was kindly supplied by Dr R. Bercks, Brunswick, Germany, and peach yellow bud mosaic virus by Dr C. E. Yarwood, Berkeley, California. Two isolates of tomato black ring virus, originally obtained from tomato and lettuce respectively, and also tobacco ring spot virus were kindly supplied by Dr K. M. Smith, Cambridge.

When these viruses were transmitted by rubbing leaves with infected sap, 'Super-floss celite' powder (Johns-Manville Ltd.) was mixed in the inoculum to increase the number of infections. Preparations of sap for serological tests were obtained by macerating either inoculated leaves covered with local lesions or systemically infected leaves. Such sap was stored frozen, and when required was thawed and clarified by centrifugation, 9000 *g* for 3 min., immediately before use.

Tobacco and *Petunia hybrida* Vilm. plants show severe symptoms when first infected, but later produce leaves that look normal although they contain virus. Such infected but normal-looking leaves were inoculated for the plant-protection tests. Plants are more susceptible in winter than in summer, and to keep them susceptible in summer they were kept well watered and shaded. All plants were grown at a mean temperature of 20°. For each protection test in tobacco, several plants were used; the left half of each normal-looking tobacco leaf was inoculated with the virus it already contained and the right halves with the virus under test. For protection tests in *P. hybrida*, each virus was inoculated on all the normal-looking leaves of several different plants.

In the serological tests, 1 ml. of suitably diluted virus preparation was mixed, in tubes of 8 mm. diameter, with 1 ml. of suitably diluted virus antiserum. Control mixtures containing normal tobacco sap or normal rabbit serum, or both, were always made up. The amount of precipitation after 5 hr. incubation at 37° was recorded. A few tests were made with an antiserum to potato bouquet virus kindly supplied by Dr R. Bercks, but most of the tests were done with antisera made by the method already described (Harrison, 1957*a*) to local-lesion isolates of beet ringspot and potato bouquet viruses. The antisera were either stored frozen or diluted with an equal volume of glycerol and kept at 2°.

RESULTS

Symptoms and host range

The symptoms caused by beet ringspot virus in tobacco, *Nicotiana rustica* L., French bean (*Phaseolus vulgaris* L. var. Prince), *Petunia hybrida* and *Chenopodium amaranticolor* Coste & Reyn. were thought to be characteristic (Harrison, 1957*a*), but potato bouquet and tomato black ring viruses also produce symptoms in all these plants very like those caused by beet ringspot virus, except that those caused by the latter are somewhat more severe in tobacco, French bean and *C. amaranticolor*. Tobacco ring spot virus and peach yellow bud mosaic virus cannot be readily distinguished from the other viruses in *P. hybrida* and French bean, but in contrast to beet ringspot virus they rarely become systemic in *C. amaranticolor* although they produce local lesions in inoculated leaves. In tobacco, peach yellow bud mosaic virus produces local lesions but, unlike beet ringspot virus, does not become systemic: although tobacco ring spot virus produces local lesions and usually becomes systemic in tobacco, the young leaves on 'recovered' plants are mottled, whereas those on similar plants infected with beet ringspot virus are not. Tobacco ring spot virus causes a more severe systemic disease than beet ringspot virus in *N. rustica*. The symptoms produced by beet ringspot virus in cucumber (*Cucumis sativus* L. var. Lockie's Perfection) and tomato (*Lycopersicon esculentum* Mill. var. Kondine Red) are like those caused by tomato black ring virus (Smith, 1946): the symptoms on cucumber include the large enations on the undersides of systemically infected leaves.

Although beet ringspot virus occurs naturally in potato and sugar beet, and potato bouquet virus occurs naturally in potato, Smith (1946) states that these two species are immune from tomato black ring virus. In my tests, all three viruses were readily transmitted to sugar beet (*Beta vulgaris* L. var. Kleinwanzleben E) by mechanical inoculation and all caused systemic infection. Potato plants are not easily infected with these viruses by mechanical inoculation, and in tests with the varieties Kerr's Pink and King Edward, beet ringspot and potato bouquet viruses infected some plants whereas tomato black ring virus (tomato and lettuce isolates) infected none. All the isolates except tomato black ring virus (tomato) infected plants of the variety Chancellor systemically. The number of plants which developed local lesions and the number which became systemically infected respectively, were 4/4 and 4/4 for beet ringspot, 4/4 and 2/4 for potato bouquet, 7/7 and 6/7 for tomato black ring (lettuce) and 4/7 and 0/7 for tomato black ring (tomato) viruses. Thus although tomato black ring virus (tomato), which is derived from Smith's original isolate of the virus, failed to infect potato systemically, plants of the variety Chancellor did become systemically infected with tomato black ring virus (lettuce).

Comparison of the lists of species found naturally infected with beet ringspot, potato bouquet and tomato black ring viruses shows that, with one exception, each virus has been found in different species. Beet ringspot virus has been isolated from sugar beet, potato, turnip, strawberry, *Crocus* and several weed species, potato bouquet virus from potato, bean and a weed, and tomato black ring virus from tomato and lettuce. Although these lists show that beet ringspot virus has many natural hosts, further work indicated that even the list for it was far from complete. Beet ringspot virus is soil-borne, and it was found to infect systemically plants of wheat, oat, French bean, peach and the cherry rootstock F 12/1 when these were grown in soil collected from a field where plants infected with beet ringspot virus were common: of the species used only barley did not become infected. It therefore seems probable that our knowledge of the natural host range of all three of these viruses has been restricted by the small number of attempts made to detect them in different species. Tobacco ring spot virus has also been found in many species (see Smith, 1957); peach yellow bud mosaic virus occurs naturally in peach (Thomas *et al.* 1944) and probably in Himalaya blackberry (Alcorn, Wilhelm & Thomas, 1955).

The thermal inactivation points of beet ringspot, potato bouquet and tomato black ring viruses have been given as 58–63°: that for tobacco ring spot virus is usually given as 63–68°.

Plant-protection tests

Thirty-one isolates of beet ringspot virus originally obtained from 15 different species growing on five farms within 40 miles from Dundee, Scotland, produced no local or systemic symptoms when inoculated to tobacco plants which were already infected with either of the stock isolates of beet ringspot virus. Similarly, the two stock isolates caused no symptoms when inoculated to plants infected with these 31 isolates. No variations among isolates of beet ringspot virus from Scotland were revealed in these tests.

Seven isolates of beet ringspot virus caused no symptoms in plants infected with potato bouquet virus but, by contrast, beet ringspot virus did not protect plants from infection by potato bouquet virus. Many chlorotic or necrotic lesions developed especially in the youngest inoculated leaves of these plants (Harrison, 1957*a*, fig. 9) and systemic symptoms appeared later. Neither the lesions in the inoculated leaves nor the systemic symptoms were as severe as in comparable healthy plants inoculated with potato bouquet virus. One possible explanation of these results was that the culture of potato bouquet virus contained a mixture of two viruses, one of which was related to beet ringspot virus. Seven local-lesion isolates of potato bouquet virus were therefore made, and each was passed through a single local lesion twice. All the local-lesion isolates protected plants completely from infection by the stock culture of potato bouquet virus, showing that the latter was not a mixture of two viruses. Further tests showed that potato bouquet virus produced the same kind of symptom in plants already infected with each of seven different isolates of beet ringspot virus.

The two isolates of tomato black ring virus protected completely against one another. The isolate from lettuce usually protected completely against beet ringspot virus: sometimes, however, faint chlorotic lesions developed in the inoculated leaves and a very mild systemic symptom developed in one plant. More clearly defined chlorotic lesions and mild systemic symptoms were produced when beet ringspot virus was inoculated to plants infected with tomato black ring virus (tomato). Beet ringspot virus appeared to protect against the tomato isolate but never against the lettuce isolate of tomato black ring virus. The latter produced symptoms in plants infected with beet ringspot virus very like those caused by potato bouquet virus, but slightly less severe. Potato bouquet virus protected against both isolates of tomato black ring virus: it caused faint chlorotic lesions and mild systemic symptoms in plants infected with the tomato isolate but only did this rarely in plants infected with the lettuce isolate.

Table 1. *Symptoms produced in the plant-protection tests*

	Symptoms in inoculated leaves already infected with					
Second virus	Beet ringspot virus	Tomato black ring virus (tomato)	Tomato black ring virus (lettuce)	Potato bouquet virus	Tobacco ring spot virus	Peach† yellow bud mosaic virus
Beet ringspot virus	0	+*	t*	0	++*	++*
Tomato black ring virus (tomato)	0	0	0	0	0*	—
Tomato black ring virus (lettuce)	++*	0	0	0	++*	++*
Potato bouquet virus	++*	+*	t*	0	++*	++*
Tobacco ring spot virus	+++*	+++*	+++*	+++*	0	+*
Peach yellow bud mosaic virus	+++	–	–	–	+++	0

Degree of local lesion formation in inoculated leaves: 0, none; t, trace; +, weak; ++, moderate; +++, strong; –, not tested.

* Systemic symptoms observed.

† Recovered *Petunia hybrida* plants used. Tests with the other viruses were made in recovered tobacco plants.

Tobacco ring spot virus produced many local lesions and systemic symptoms in plants already infected with beet ringspot, potato bouquet or tomato black ring viruses: also, plants infected with tobacco ring spot virus were susceptible to these three viruses and to peach yellow bud mosaic virus. The latter readily infected plants containing beet ringspot virus. Because peach yellow bud mosaic virus does not infect tobacco systemically, reciprocal tests were made in infected *Petunia hybrida* plants which had 'recovered'. These plants were readily infected with beet ringspot, potato bouquet and tomato black ring viruses, and less readily by tobacco ring spot virus, which was slow to produce systemic symptoms. The results of these plant-protection tests are summarized in Table 1.

Other tests in tobacco showed that beet ringspot virus did not protect against raspberry ring spot, potato stem mottle or lucerne mosaic viruses, potato bouquet virus did not protect against raspberry ring spot or potato stem mottle viruses, tobacco ring spot virus did not protect against raspberry ring spot virus, and lucerne mosaic virus did not protect against beet ringspot, potato bouquet or raspberry ring spot viruses.

An experiment of the type described by Holmes (1956) was made using beet ringspot, potato bouquet and tobacco ring spot viruses. Groups of plants were inoculated with each virus and with preparations containing each possible pair of viruses. The effects of the viruses on the growth of the plants after recovery were recorded. Plants infected with the mixture of tobacco ring spot + beet ringspot viruses were most stunted and those with potato bouquet virus least stunted. The degree of stunting increased in the order:

potato bouquet virus = potato bouquet virus + beet ringspot virus
< beet ringspot virus < tobacco ring spot virus
< tobacco ring spot virus + potato bouquet virus
< tobacco ring spot virus + beet ringspot virus.

The ability of potato bouquet and beet ringspot viruses to enhance the stunting produced by tobacco ring spot virus and the failure of potato bouquet virus to enhance the stunting caused by beet ringspot virus suggest that the two latter are related virus strains, but that neither is related to tobacco ring spot virus. Twelve weeks after infection with the mixture of potato bouquet + beet ringspot viruses, sap from the 'recovered' leaves of the plants was inoculated to *Chenopodium amaranticolor*. Nearly all the lesions were of the potato bouquet virus type but a few were characteristic of the lesions produced by beet ringspot virus. Potato bouquet virus therefore seems to have suppressed almost completely the multiplication of beet ringspot virus in the systemically infected leaves of plants which received a mixed inoculum.

Serological tests

In the first series of experiments, virus antisera diluted 1/35 were mixed with graded dilutions of infective sap. Antiserum to beet ringspot virus only precipitated beet ringspot virus but antiserum to potato bouquet virus precipitated potato bouquet virus and both isolates of tomato black ring virus. Precipitation end-points of sap were usually 1/16. When used diluted 1/16 or less, however, both antisera precipitated all four virus isolates. These results suggested that the viruses were serologically related but that different pairs had different numbers of antigens in common. To estimate the extent of the serological relationship a second series of experiments was done in which the infective saps diluted 1/5 were mixed with graded dilutions of the antisera. The results are summarized in Table 2.

Potato bouquet virus and the two isolates of tomato black ring virus appear to share at least half their antigens, for antiserum to potato bouquet virus had the same precipitation end-point against all three. In an attempt to detect antigenic differences between potato bouquet and tomato black ring viruses, antiserum to potato bouquet virus was absorbed with tomato black ring virus (lettuce) before testing. One ml. antiserum was incubated for 3 hr. at 37° with 7 ml. sap containing tomato black ring virus (lettuce), the mixture kept overnight at 2°, centrifuged and the clear supernatant fluid used. The precipitation end-point of this preparation was much lower than that of the untreated antiserum, but the same whether measured using potato bouquet or tomato black ring (lettuce) viruses. There is, therefore, little or no antigenic difference between these two viruses.

Beet ringspot virus, by contrast, seems to share less than 20 % of its antigens with potato bouquet and tomato black ring viruses. The precipitation end-points of beet ringspot virus antiserum against potato bouquet and tomato black ring viruses were the same, but scarcely greater than that against normal tobacco sap. Despite the small end-point, antibodies to the virus were present, for after the serum was absorbed with normal tobacco sap, it still precipitated potato bouquet virus. In the same way it was shown that antiserum to potato bouquet virus had a low but real precipitation end-point against beet ringspot virus. Also, when potato bouquet virus was incubated with antiserum to beet ringspot virus for 3 hr. at 37° and centrifuged, no infectivity remained in the supernatant fluid: by contrast, a preparation of potato bouquet virus similarly treated with normal serum was highly infective. Neither the antiserum to beet ringspot virus nor the antiserum to potato bouquet virus precipitated tobacco ring spot virus, although highly infective virus preparations were used.

Table 2. *Precipitation end-points against different ringspot viruses of virus antisera*

	Reciprocal of precipitation end-point				
	Beet ringspot virus antiserum		Potato bouquet virus antiserum		
Antiserum tested against	Untreated	Absorbed with 3 vol. normal tobacco sap	Untreated	Absorbed with 3 vol. normal tobacco sap	Absorbed with 7 vol. undiluted tomato black ring virus (lettuce) preparation
Beet ringspot virus	128	128	16	16	—
Potato bouquet virus	16	8	128	128	16
Tomato black ring virus (lettuce)	16	—	128	—	16
Tomato black ring virus (tomato)	16	—	128	—	—
Tobacco ring spot virus	—	0 (4)	—	0 (4)	—
Normal tobacco sap	8–16	0 (4)	4–8	0 (4)	0 (8)

Precipitation end-points of the antisera were recorded after 5 hr. incubation at 37°. The figures in parentheses are the smallest dilutions of antiserum tested. The virus preparations consisted of infective tobacco sap diluted 1/5. Absorption was done by incubating the mixture for 3 hr. at 37°, keeping overnight at 2° and clarifying by centrifugation before use.

DISCUSSION

The results of the serological and plant-protection experiments show that beet ringspot, potato bouquet and tomato black ring viruses should be regarded as strains of one virus. As tomato black ring virus (tomato) seems to have been the first of the strains to be named, beet ringspot and potato bouquet viruses are probably best considered as strains of tomato black ring virus. The isolates of tomato black ring virus from tomato and lettuce, and potato bouquet virus are closely related strains but beet ringspot virus is more distantly related to these three. The degree of relationship between the different isolates is represented by the series:

potato bouquet virus—tomato black ring virus (lettuce)—tomato black ring virus (tomato)————beet ringspot virus,

and is reflected in the slight difference between the symptoms caused by beet ringspot virus and the other strains. There was no evidence of strain relationship between any of this group and tobacco ring spot virus or peach yellow bud mosaic virus.

Ringspot viruses with the most antigens in common were usually also the ones that appeared most closely related in the plant-protection experiments, a conclusion similar to that reached by Matthews (1949) from studies on potato virus *X*. Detailed examination of the results from the two kinds of tests, however, shows that the correlation between them is not complete. This is seen in the following examples. Reciprocally protecting virus strains can be antigenically distinguishable by cross-absorption tests (potato virus *X*; Matthews, 1949) or indistinguishable (isolates of beet ringspot virus) from one another. Similarly, incompletely protecting virus strains can be antigenically distinguishable (potato bouquet and beet ringspot viruses) or indistinguishable (potato bouquet and tomato black ring viruses) from one another. That protection should not be complete between two viruses which are antigenically indistinguishable is especially interesting but it should be remembered that very small differences in antigenic constitution might not be detected in the experiments described above. Whether or not very slight antigenic differences exist between potato bouquet and tomato black ring viruses, the comparisons made above seem to provide new evidence for the view that serological and plant-protection tests measure different properties of the virus particles. The relative value of the two kinds of tests for establishing virus relationships has been fully discussed by Bawden & Kassanis (1951) and need not be further considered here.

Non-reciprocal protection is not a new phenomenon, but it happened unusually often in my tests, perhaps partly because the virus-host system favoured observing it. When testing possible relationships between viruses by plant-protection tests it is obviously important to do reciprocal tests whenever possible. When protection is incomplete, as between beet ringspot and potato bouquet viruses, the lesions which develop after inoculating leaves containing beet ringspot virus with potato bouquet virus are much less severe than those caused by potato bouquet virus in previously healthy leaves. From the size of the potato bouquet virus lesions in leaves containing beet ringspot virus, it can be deduced that changes, probably the multiplication of potato bouquet virus, occurred in a hundred or more cells in each lesion, in all of which beet ringspot virus had presumably multiplied before the leaves were inoculated with potato bouquet virus. Possible explanations for the poor development of the potato bouquet virus lesions are that the virus invaded fewer cells in each lesion than usual, that it multiplied less than usual in such cells or that the cells were partially protected from the usual deleterious effects of multiplication of potato bouquet virus. The ability to multiply and cause symptoms in cells in which a serologically related virus has already multiplied seems to be possessed to different degrees by different virus strains, and the strains of tomato black ring virus can be arranged in order of decreasing ability to do this:

potato bouquet—tomato black ring (lettuce)—beet ringspot—tomato black ring (tomato).

Because infectivity seems to be a property of the nucleic acid part of tobacco mosaic virus (Fraenkel-Conrat, 1956; Gierer & Schramm, 1956) and possibly of other viruses, it is tempting to speculate that some property of the virus nucleic acid determines the result of plant-protection tests. Whether or not it is possible for the properties of virus nucleic acid to vary in different virus strains independently of variations in virus antigenicity is unknown, but if such variations do occur they may cause the discrepancies in estimates of the degree of relationship between virus strains as determined by the results of plant-protection and serological experiments.

Presumably viruses evolve from forms with all their antigens in common to those with but few in common, and finally into types in which no common antigens can be detected and which would be described as separate viruses. A parallel series of reactions in the plant-protection tests can be divided into four stages: (1) Complete and reciprocal protection; for example, lettuce and tomato isolates of tomato black ring virus. (2) Non-reciprocal protection; tomato black ring (lettuce) and beet ringspot viruses. (3) Partial protection, with infection being reciprocal but the symptoms caused by the second virus being milder than usual; potato bouquet virus and the pseudo-aucuba strain (Bercks & Gehring, 1956). (4) No protection; tobacco ring spot and potato bouquet viruses. Divergence between strains will probably be more gradual than suggested by these four categories: for instance, a relationship might appear to be of stage 3 type when tests are done in one set of conditions or in one host, and of stage 2 type in another. Also, all gradations between stages 3 and 4 are possible.

There seems good evidence that tomato black ring virus has evolved rather differently in different countries. Isolates of the beet ringspot virus type from different localities in Scotland were indistinguishable and the differences between the two English tomato black ring virus isolates were very slight. The German isolate (potato bouquet virus) was just distinguishable from the English isolates and readily distinguishable from the Scottish ones: the English isolates were rather less distinct from the Scottish isolates than was potato bouquet virus. Thus the greatest differences were between isolates from the most widely separated sources, with differences becoming smaller with decreasing distance between sources until isolates found in localities only a few miles apart were indistinguishable. When still greater distances are involved, such as those between the sources of the European and North American ringspot viruses used, the differences were greater than those between related virus strains: this last point, however, is at the most only suggestive, for it is by no means certain that strains of tomato black ring virus do not occur in North America. There is some suggestion that the same kind of geographical variation occurs in other soil-borne viruses. For instance, there are considerable antigenic differences between potato stem mottle virus found in Holland and a form found in Scotland (Cadman & Harrison, unpublished), and although this virus has not been found in North America, the soil-borne wheat mosaic virus occurring there has particles of a similar shape and size (Gold, Scott & McKinney, 1957) and may be a representative of the same major virus group. Here too, the differences between the European types are almost certainly much smaller than those between European and North American types. Although it is possible that soil-borne viruses are particularly prone to geographical variation, perhaps because they have less chance than other viruses of becoming widely disseminated, adequate comparisons must be made with variation in viruses which have flying vectors before such a view can be accepted or refuted. If more geographical variation occurs with soil-borne than with other viruses, and if viruses of the ringspot type are commonly soil-borne as has been suggested (Harrison, 1956), then the existence of many apparently distinct viruses that produce similar symptoms of the ringspot type (see Smith, 1957) is only to be expected.

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‘Restoration’ of *Escherichia coli* strain B After Irradiation: its Dependence on Suboptimal Growth Conditions

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SUMMARY: After irradiation of *Escherichia coli* strain B by ultraviolet or ionizing radiations, different plating media give rise to widely different viable counts; the surviving fractions are smallest on media which are optimal for the growth of unirradiated organisms. It is concluded that inhibition of colony formation after irradiation is due in part to an injury which leads to imbalance in the synthetic processes of the cell, so that ‘recovery’ or ‘restoration’ may be brought about by suboptimal conditions of growth. The proportion of cell deaths which may be ascribed to this particular injury is determined by the conditions obtaining in bacterial suspensions before and during irradiation, and also by the type of radiation used.

There are numerous papers in the literature dealing with modification of radiation damage to micro-organisms by treatment both before and after irradiation. A number of these investigations have been made with *Escherichia coli* strain B, which appears to be atypical in some respects in its response to radiation, and presumably also in its biochemical behaviour. ‘Survival’ of micro-organisms after irradiation is commonly defined as the ability to form visible colonies on the surface of solid media; the extent to which *E. coli* B survives, after a given dose of radiation, has been reported as varying with the interval of time between irradiation and plating, the ‘incubation medium’ before plating, the temperature of incubation after irradiation, and the plating medium (e.g. Roberts & Aldous, 1949; Stein & Meutzner, 1950; Heinmets, Lehman, Taylor & Kathan, 1954). The investigation to be described has been concerned mainly with the effect of the plating medium on the observed survival of *E. coli* B. The results suggest that a single mechanism may underlie the varied means which may be used after irradiation to modify the response of this micro-organism.

METHODS

Media and chemicals. Oxoid ‘Blood Agar Base’ and Difco ‘Nutrient Agar’, both obtained as dehydrated granules, were used as plating media. When reconstituted according to the makers’ instructions, the former contained: 10 g./l. Lab-lemco meat extract, 10 g./l. Oxoid peptone, 5 g./l. NaCl, and 15 g./l. agar-agar; the latter contained 3 g./l. Bacto-beef extract, 5 g./l. Bacto-peptone, and 15 g./l. agar-agar. Oxoid Nutrient Broth and Difco Nutrient Broth were also used, which were of the same composition, apart from the agar-agar, as Oxoid Blood Agar Base and Difco Nutrient Agar respectively. The pH values were 7·2 and 6·8 respectively.

Oxoid NaCl (Bacteriological grade) and Oxoid Peptone (papain digest of meat proteins) were used for investigations with these media constitutents. The pH value of Oxoid peptone solution at 10 g./l. was 5·6.

Buffer solutions were made from chemicals of analytical reagent quality in glass-distilled water. Adjustment of pH value was made by adding acetic acid or sodium hydroxide. Tests showed that the addition of these compounds to the media was without effect on irradiated bacteria except in so far as they changed the pH value.

Organisms used. Escherichia coli strain B; *E. coli* strain B/r; *E. coli* strain K12 (W 1485: methionine-requiring); *E. coli* strain '86'; *Shigella flexneri* strain Y6R; *Salmonella typhimurium* strain LT2.

Preparation of bacteria for irradiation. Stocks of organisms were routinely maintained on slopes of Oxoid nutrient agar, and subcultured into Oxoid nutrient broth, which was incubated overnight without aeration when bacteria in the stationary phase were required. The yield from such cultures was 10^8–10^9 organisms/ml. When suspensions of bacteria in the logarithmic phase were required, these were obtained by growing the organisms for short periods at 37° under the moderate aeration provided by the rotation of McCartney bottles on a turntable. 0·2 ml. of an overnight broth culture was inoculated into 5 ml. broth, which was incubated in this way for 1·5 hr. From this culture 0·2 ml. was inoculated into a fresh McCartney bottle containing 5 ml. broth, which was rotated in the incubator for 1·7 hr. The suspensions were centrifuged and washed three times in 0·067 M-phosphate buffer (pH 6·9) and finally resuspended in buffer. Suitable dilutions of organisms for irradiation were made with this phosphate buffer, the maximum concentration used for u.v.-irradiation being 6×10^5 organisms/ml. With X-rays more concentrated suspensions (up to 10^7 organisms/ml.) were used when it was desired to follow survival down to 10^{-4} of the controls.

Plating. Comparisons between different media were made by dispensing samples of suitable dilution from the same irradiated or control suspensions on to agar plates and spreading the fluid over the surface. The samples were delivered by dropping pipettes tipped with platinum tubing, external diameter 0·95 mm., drop size 0·017 ml. A sufficient number of drops, up to a maximum of 25, was delivered to give a count of 100–200 colonies/plate. Two or more plates were used to determine the survival after each dose of radiation.

In some experiments volumes of control and irradiated suspensions were delivered to the surface of pieces of cellophan, about 4 sq.cm. in area, which had been laid on the surface of the media under test. As it was convenient to add only small volumes to the cellophan, these volumes were applied by means of 'Agla' microsyringes (Burroughs Wellcome and Co. London), used without needles. The drops, varying from 0·001 to 0·005 ml. in size as required, came away cleanly when the pieces of cellophan were touched to the ground-glass tips of the syringes, which had been treated with silicone grease. The drops were spread to within 2 or 3 mm. of the edge of the cellophan. After incubation on solid medium colony counts on the cellophan corresponded, volume for volume, with those made directly on the agar surfaces. The standard deviation for any count was the same with both techniques, about $\surd 2n$ for a count of n colonies; the confidence limits indicated in the diagrams represent twice the standard error in each count.

Methods and sources of irradiation. The absolute doses of radiation received by the suspensions were not determined very accurately, as experiments usually involved comparisons of viable counts on different media for samples taken from the same vessel. Radiation dose rates were constant throughout all experiments. Comparisons of the effects of different treatments before or after irradiation were usually made by studying 'dose effect' or 'survival' curves.

Ultraviolet radiation. The source was a water-jacketed Hanovia 'Germicidal' lamp, 15 W., with 85 % of the emitted energy at 2537 Å. (maker's figure). Irradiations were carried out in a standard position, 74 cm. from the lamp, the energy being 3·3 ergs/mm.^{2}/sec., as measured by a photocell very kindly calibrated by Dr R. Latarjet. Suspensions were irradiated from above in open glass vessels with optically flat bases, for times varying from 6 sec. to 4·5 min. When cultures were compared which had been treated differently before irradiation, they were irradiated side by side in identical vessels.

X-rays. A 'Stabilivolt' set, with a Philips 'Metalix' tube was used at 200 kV, 6 mA, with external filtration of 2 mm. Al and 1 mm. glass. Since sensitivity to ionizing radiation is markedly affected by oxgen concentration, the gas bubbling technique of Ebert (1955) was always used. During irradiation either oxygen or oxygen-free nitrogen was passed in a stream of fine bubbles through the suspensions, which had previously been equilibrated with the appropriate gas. The special type of vessel used has previously been described (Alper, 1955; Howard-Flanders & Alper, 1957). The dose rate in the vessel was 2000 rads/minute as determined by ferrous-ion actinometry (Miller & Wilkinson, 1952).

Neutrons. These were produced by a Van de Graaff generator, from the reaction of 1·8 MeV. deuterons with a beryllium target. The generator and the methods of dosimetry have been described by Howard-Flanders (1956) and the details of the method of irradiating bacterial suspensions by Howard-Flanders & Alper (1957).

RESULTS

In the course of an investigation on the sensitivity of *Escherichia coli* strain B to X-rays, it was found that viable counts after given doses of radiation were higher on Difco Bacto-nutrient agar than on Oxoid Blood Agar Base, the difference in number increasing with dose; thus the radiosensitivity appeared greater when the Oxoid medium was used. Colony counts on the control (unirradiated) suspensions were the same on the two media, but the colonies on the Oxoid Blood Agar Base were considerably larger and of different morphology. Differences in the slopes of survival curves were found also with other types of radiation, viz. neutrons and u.v.-radiation, but not after treatment by exposure to a temperature of 52° (Fig. 1) or to 0·6% (w/v) phenol. As shown by Figs 2–4, the ratios of the slopes of survival curves after irradiation varied with the type of radiation, the age of the culture, and, when ionizing radiation was used, with the oxygen concentration in the suspending buffer (Alper & Gillies, 1957).

Initially it was thought that the underlying phenomenon might be a radiation-induced biochemical need, which was supplied by constituents of the Difco medium, but not by the Oxoid medium. This would have accorded with some aspects of the interpretation placed on their results by Stapleton, Sbarra & Hollaender (1955), working with *Escherichia coli* B/r, and by Ellison, Erlanger & Allen (1955), who found that the addition of sodium acetate to the plating medium increased the viable counts after irradiation of *E. coli* B and *Corynebacterium bovis*.

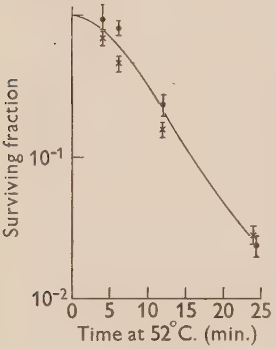

Fig. 1

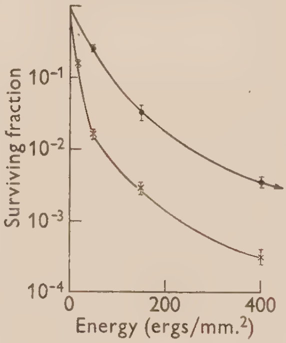

Fig. 2

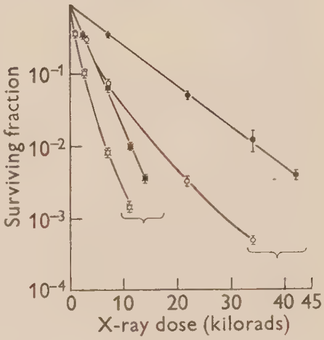

Fig. 3

Fig. 1. Survival of *Escherichia coli* strain B (stationary phase) after exposure to temperature of 52° C. ×, Oxoid agar; ●, Difco agar.

Fig. 2. Survival of *Escherichia coli* strain B (stationary phase) after exposure to ultraviolet light. ×, Oxoid agar; ●, Difco agar.

Fig. 3. Survival of *Escherichia coli* strain B (stationary phase) after exposure to X-rays under oxygenation and under anoxia. ●, Irradiated under anoxia, plated on Difco agar; ○, irradiated under anoxia, plated on Oxoid agar; ⌘, irradiated under oxygenation, plated on Difco agar; ☐, irradiated under oxygenation, plated on Oxoid agar.

However, there was no increase in the surviving fraction on Oxoid Blood Agar Base supplemented with yeast extract, whole blood, or fractions prepared from the constituents of the Difco nutrient agar. On the contrary, viable counts on the Difco nutrient agar were decreased when it was supplemented by Oxoid broth granules, or by a dialysate prepared from these. The residues of Oxoid broth after dialysis were set with agar, and viable counts on this medium approximately equalled those on unsupplemented Difco nutrient agar (Fig. 5). The increased sensitivity made evident by plating on the Oxoid Blood Agar Base could therefore be regarded as a positive action on the irradiated organisms of one or more dialysable constituents of that medium, so that their absence from the Difco medium promoted 'recovery'.

Dialysable substances effective in decreasing viable counts after irradiation

As the Oxoid Blood Agar Base contains 5 g. NaCl/l., the effects of adding NaCl to Difco nutrient agar were investigated. When it was found that addition of NaCl decreased the viable counts after irradiation, the effectiveness of the cation and anion were tested separately. The addition of 0·085 M-Na_2HPO_4/KH_2PO_4 (pH 7·0) to Difco nutrient agar was without effect. Chloride ion, on the other hand, was equally effective when introduced in equimolar concentration as NH_4Cl, KCl or NaCl (Fig. 6).

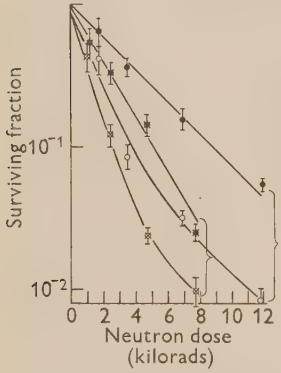

Fig. 4

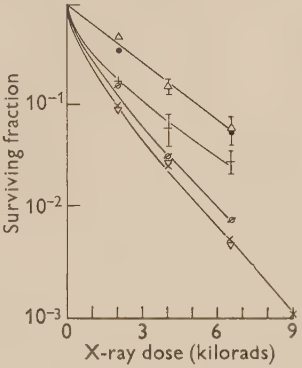

Fig. 5

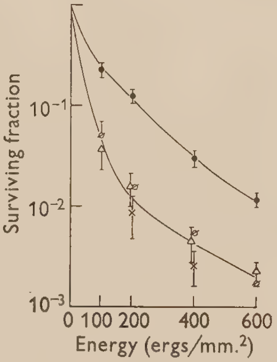

Fig. 6

Fig. 4. Survival of *Escherichia coli* strain B (stationary phase) after exposure to neutrons under oxygenation and under anoxia. (12 % of the radiation dose was contributed by X-rays.) ●, Irradiated under anoxia, plated on Difco agar; ○, irradiated under anoxia, plated on Oxoid agar; ✖, irradiated under oxygenation, plated on Difco agar; ⊗, irradiated under oxygenation, plated on Oxoid agar.

Fig. 5. Survival of *Escherichia coli* strain B (stationary phase) after exposure to X-rays under oxygenation. ●, Difco agar; +, Difco agar + NaCl, 5 g./l.; ▽, Difco agar + Oxoid broth granules, 25 g./l.; ∅, Oxoid agar + Difco broth granules, 8 g./l.; △, Oxoid broth, residue after dialysis, set with agar-agar; ×, Oxoid agar.

Fig. 6. Survival of *Escherichia coli* strain B (stationary phase) after exposure to ultraviolet light. ●, Difco agar; △, Lab-lemco, 10 g./l. + peptone 10 g./l. + KCl (0·085 M); ∅, Lab-lemco, 10 g./l. + peptone 10 g./l. + NH_4Cl (0·085 M); ×, Oxoid agar, i.e. Lab-lemco 5 g./l. + peptone 10 g./l. + NaCl (0·085 M).

However, the total action of the Oxoid Blood Agar Base could not be accounted for by chloride alone (Fig. 5). A dialyzable factor was found to be associated with peptone, which was present in higher concentration in the Oxoid than in the Difco medium (Fig. 7). When both peptone 10 g./l. and NaCl 5 g./l. were added to the Difco nutrient agar, and the pH value raised to that of Oxoid Blood Agar Base, survival curves were the same as those observed by plating on the Oxoid medium. The active factor dialysable from peptone has not yet been identified.

The effect of hydrogen-ion concentration

Since the Oxoid Blood Agar Base was at a higher pH value than the Difco nutrient agar, this parameter was investigated. The results of two experiments have been combined in Fig. 8, which shows the surviving fraction, after

50 ergs/mm.^{2} of u.v. radiation, for samples plated on: (1) Difco nutrient agar, at its usual concentration of 8 g. nutrients/l.; (2) Difco nutrient agar, with nutrients at 20 g./l. (to match the concentration of nutrients in the Oxoid medium); (3) Difco agar, with 8 g. nutrients/l. + Oxoid peptone 10 g./l.; (4) Difco agar, with nutrients at 8 g./l. + NaCl, 5 g./l.; (5) Oxoid Blood Agar Base (nutrients 20 g./l., NaCl 5 g./l.). The surviving fractions differed somewhat in the two experiments, so they have been combined for presentation in one figure by displacing the ordinate scale applying to the second experiment so that the two Difco nutrient agar curves coincide. It can be seen that for all the media tested, the surviving fraction decreased with increasing pH value. The curve for Difco nutrient agar + extra peptone is parallel with that for ordinary Difco nutrient agar, showing that the enrichment by peptone decreased the surviving fraction by a constant fraction, whatever the pH value. The increase in the surviving fraction on the Oxoid media at high pH values may be associated with the fact that precipitates formed in these on autoclaving, with a possible removal of limiting nutritional factors. The control counts on these two media were lower than on all the others, which did not differ from each other significantly. Control counts on the Oxoid media at pH 8·1 and 8·8 were respectively 61 and 42 % of the mean for all other media. The control counts observed on the two media in question were used in estimating the surviving fraction.

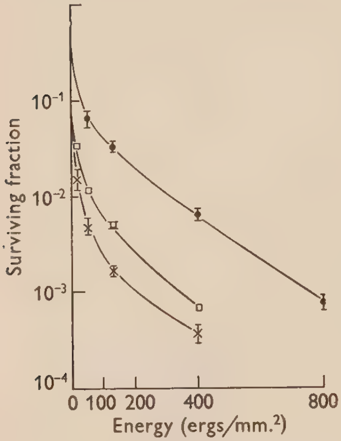

Fig. 7

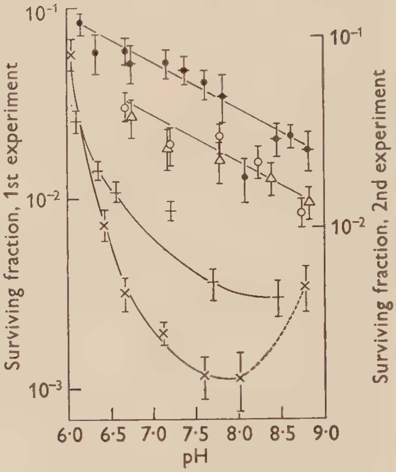

Fig. 8

Fig. 7. Survival of *Escherichia coli* strain B (logarithmic phase) after exposure to ultraviolet light. ●, Difco agar; □, Difco agar + dialysate of peptone, pH 7·3; ×, Oxoid agar.

Fig. 8. Variation with pH in surviving fraction of *Escherichia coli* strain B (logarithmic phase) after exposure to 50 ergs/mm.^{2} ultraviolet light. 1st *experiment*: ●, Difco nutrient broth, 8 g./l. + agar-agar; +, Difco nutrient broth 8 g./l. + NaCl, 5 g./l. + agar-agar; ×, Oxoid nutrient broth + agar-agar. 2nd *experiment*: -●-, Difco nutrient broth, 8 g./l.; ○, Difco nutrient broth + peptone, 10 g./l.; △, Difco nutrient broth, 20 g./l.

The dependence of the survival of irradiated *Escherichia coli* strain B organisms on the pH value of the plating medium was reported by Weatherwax (1956), who also found that the survival was considerably greater on acid than on alkaline media.

Figure 9 illustrates full survival curves for bacteria which had been harvested in the stationary phase of growth. The highest survival was observed on Difco nutrient agar at pH 6·15 although it was supplemented with Oxoid peptone 10 g./l. On this medium the colonies grew very slowly and were large enough to count only after incubation for 60 hr. at 37°.

The possibility of a genetic origin for the effect of medium on viable count after irradiation

Since the Oxoid Blood Agar Base was used for the routine culture of *Escherichia coli* strain B, it was necessary to examine whether some form of selection might be responsible for the differences in viable counts after irradiation. The strain was carried through six passages on Difco Bacto-agar and Difco Bacto-nutrient broth; irradiation of these cultures by X-rays or ultraviolet light gave the same results as those observed with routine cultures. The possibility was envisaged that a radiation-induced mutation of a biochemical nature might express itself as inhibition of colony formation on the Oxoid Blood Agar Base. After a dose of *c.* 6000 rads of X-rays, there were 10 % survivors on Difco nutrient agar, but only 1 % on Oxoid Blood Agar Base. Difco nutrient agar plates bearing about 150 colonies each, which represented this surviving fraction, were replicated by the technique of Lederberg & Lederberg (1952) on to fresh Oxoid Blood Agar Base plates. If the differences between viable counts on the two media were due to the induction of mutants unable to grow on the Oxoid medium, it might be expected that only about 15 colonies/Difco nutrient agar plate would be replicated on the Oxoid medium. However, every colony on the Difco plates was reproduced on Oxoid medium.

The time involved in inhibition of colony formation by chloride and the peptone factor

Since irradiated bacteria seeded on Difco nutrient agar gave rise to daughters able to form colonies on Oxoid Blood Agar base, though the irradiated bacteria themselves could not do so, it was of interest to see at what stage this transition occurred. Samples of control and irradiated suspensions were plated on cellophan squares laid on the surfaces of Difco or Oxoid nutrient agar plates previously warmed to 37°. At given times thereafter, squares were transferred from the initial plates to fresh plates of the same medium and the other medium. The squares transferred soon after plating gave rise, after incubation, to colony counts typical of the second medium, while those which were transferred late gave rise to counts typical of the first medium; intermediate transfers gave rise to intermediate counts, as shown by Fig. 10. Simultaneously with the transfers, the rate of growth in these conditions was studied by removing pieces of cellophan at intervals, shaking vigorously in phosphate buffer and making viable counts. The experiment illustrated by Fig. 10 was carried out at 32° in order to prolong the lag phase. The period during which the colony count could change on transfer appeared to last until the first division had taken place; in some experiments changes occurred even after this, particularly with the transfers from Oxoid to Difco nutrient agar. Weatherwax (1956) carried out analogous experiments, in which changes in the pH value of the medium were effected at various times after plating. This author considered that the colony-forming ability of irradiated cells was determined at about the time of their first division. It is obviously of great importance to determine the precise time after which change can no longer occur; further experiments under more rigorous conditions are planned.

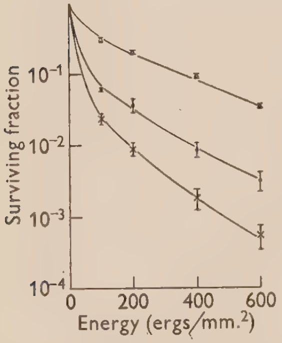

Fig. 9

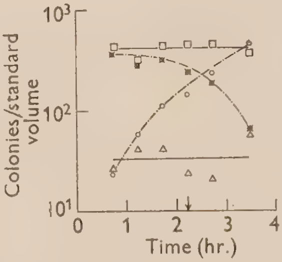

Fig. 10

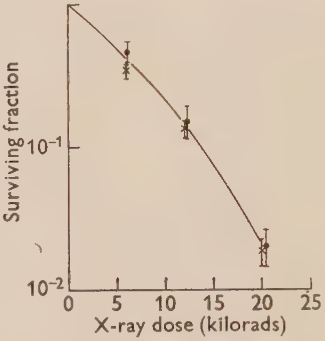

Fig. 11

Fig. 9. Survival of *Escherichia coli* strain B (stationary phase) after exposure to ultraviolet light. ●, Difco agar, pH 6·8; △, Difco agar + peptone, 10 g./l., pH 6·1; ×, Lab-lemco 10 g./l. + peptone 10 g./l., pH 7·5.

Fig. 10. The effect of transferring *Escherichia coli* strain B (stationary phase) from one medium to another at varying times after 2000 rads of X-rays under oxygenation. Plates incubated at 32° C. □—□, Bacteria seeded on Difco agar plates, transferred to fresh Difco agar plates; △—△, bacteria seeded on Oxoid agar plates, transferred to fresh Oxoid agar plates; ○·—·○, bacteria seeded on Difco agar plates, transferred to fresh Oxoid agar plates; ⊠·—·⊠, bacteria seeded on Oxoid agar plates, transferred to fresh Difco agar plates. The arrow points to the end of lag phase on Oxoid at 32°.

Fig. 11. Survival of *Escherichia coli* K12 (methionine-requiring variant) after exposure to X-rays under oxygenation. ●, Difco agar; ○, Oxoid agar.

Incubation of plates at temperatures near 30° resulted in rather smaller surviving fractions than those observed from plates incubated at 37°, in agreement with the observations on *Escherichia coli* strain B made by Anderson (1951).

Other bacteria. Various other strains were tested for survival on the Difco and Oxoid media after irradiation. Equal viable counts on the two media were obtained with *Escherichia coli* strain B/r, *E. coli* 86, *Shigella flexneri* Y6R, and a methionine-requiring strain of *E. coli* K12, all in the stationary stage. The results with the last mentioned organism were of particular interest. As both media were 'complete', it was thought immaterial that the organisms were unable to synthesize methionine. As it happened, the strain grew on the Oxoid Blood Agar Base at a rate comparable with the other *E. coli* strains used; on the Difco nutrient agar, however, the growth was very slow and colonies could be counted only after 48 hr. of incubation. Nevertheless, the survival curves from counts on the two media were identical (Fig. 11).

A report on the effect of the plating medium on the numbers of biochemical mutants observed after u.v.-irradiation of *Salmonella typhimurium* (Witkin, 1956) prompted a trial of this organism. The radiosensitivity observed by plating on Oxoid Blood Agar Base was larger than on Difco nutrient agar, the ratios of the slopes of the survival curves being 1·7. With *Escherichia coli* strain B irradiated in comparable conditions, the ratios of the slopes were about 6.

DISCUSSION

The general conclusion which can be drawn from these results is that conditions which are optimal for the growth of unirradiated *Escherichia coli* strain B organism are those in which radiation damage, as estimated by viable counts, has its maximum effect. This conclusion is supported by several independent lines of evidence: (*a*) Preliminary experiments have shown that both unirradiated and irradiated *E. coli* B have a shorter lag phase and a shorter generation time in Difco broth to which NaCl has been added than in the unsupplemented Difco broth. (*b*) A greater degree of survival was observed on media of low pH value, on which colonies were observed to develop slowly. (*c*) The increased effectiveness of radiation observed on media containing chloride and/or extra peptone was not maintained at the higher radiation doses. Survival curves changed in slope and tended to run parallel, particularly after u.v.-irradiation, showing that the survivors of the larger irradiation doses were not sensitive to the action of factors in the medium. The change in slope of the survival curve is probably associated with the increased length of lag phase which radiation is well-known to induce in micro-organisms. (*d*) The action of the factors in the medium was much more pronounced with logarithmic than with stationary-stage bacteria; the latter, as is well known, take longer to go into division after re-inoculation.

Nevertheless, not all conditions which slowed growth could protect *Escherichia coli* strain B organisms after irradiation against factors in the medium; incubation of this strain at suboptimal temperatures did not promote 'recovery', in contrast to the observations made by Stapleton, Billen & Hollaender (1953) on other strains of *E. coli*. Furthermore, with other micro-organisms the imposition of sub-optimal conditions did not necessarily bring about 'recovery', as shown by the experiment with *E. coli* K12.

Numerous investigations have been reported which deal with modification of the radiation response of micro-organisms by the post-irradiation treatment. The majority of these have shown evidence of 'restoration' or 'recovery' in conditions which would normally be regarded as suboptimal for the growth of the organisms (Latarjet, 1943; Roberts & Aldous, 1949; Stein & Meutzner, 1950; Anderson, 1951; Stapleton *et al.* 1953; Buzzell, 1956; Barner & Cohen, 1956), or else in conditions in which a preliminary incubation period was allowed in a medium which permitted some metabolism to occur, but did not provide all the nutrients required for division (Wainwright & Nevill, 1955; Heinmets *et al.* 1954). There appear to be only a few investigations reported in which 'restoration' was associated with the provision of extra metabolism in the planting medium (Stapleton *et al.* 1955; Ellison *et al.* 1955). The experiments of Stapleton *et al.* with *Escherichia coli* strain B/r cannot be interpreted directly as indicating that the plating medium supplied a biochemical need induced by irradiation. Bacteria which had been grown in nutrient broth showed greater survival when plated on nutrient than on minimal agar, but the extent of the survival of these bacteria was no greater than that of bacteria grown in minimal liquid medium, before irradiation, and plated afterwards on minimal agar. In the experiments of Ellison *et al.* (1955) 'recovery' was obtained with *E. coli* B and *Corynebacterium bovis* when sodium acetate was introduced into the plating medium, a result which the authors interpreted as indicating reversal of radiation damage by this compound. However, they observed that colony growth was slower on the media which contained acetate, which suggests the possibility that their results may have been another manifestation of the association of 'restoration' with suboptimal conditions of growth.

In some of the investigations in which *Escherichia coli* strain B has been used, certain procedures applied after irradiation have been regarded as promoting restoration per se; for example, incubation at temperatures higher than 37° (Stein & Meutzner, 1950; Anderson, 1951; Buzzell, 1956); incubation in a basal medium before plating on nutrient agar (Barner & Cohen, 1956). However, the phenomenon of restoration may perhaps be described with a somewhat different emphasis if it be considered that such procedures before plating are of a nature to slow down metabolism and so protect the irradiated bacteria against the action of the peptone factor in the plating medium, and the chloride, where this was added (Buzzell, 1956; Barner & Cohen, 1956). Barner & Cohen observed an increase in viable counts with u.v.-irradiated *E. coli* B, when successive samples from a minimal liquid medium were plated on Difco nutrient agar containing 5 g. NaCl/l. This can be regarded as analogous with our transfer experiments (Fig. 10), in which a rise in viable count was observed during the first few hours after plating when cellophan squares were transferred from Difco nutrient agar to Oxoid Blood Agar Base; bacteria transferred early were susceptible to the action of NaCl and the peptone factor, but later they were no longer affected by these substances.

It has been suggested by various authors that the failure of micro-organisms to form colonies after irradiation may be attributed in part to an imbalance in their synthetic processes (Heinmets & Kathan, 1954; Heinmets *et al.* 1954; Latarjet, 1954; Barner & Cohen, 1956). Laterjet (1954) described restoration by certain procedures, such as the action of visible light, as 'restoration by superimposed injury', the latter being of a nature to slow up certain metabolic processes and so prevent the imbalance from occurring. Our results with *Escherichia coli* strain B support the view that growth of an unbalanced nature is responsible for a part of the lethal effects of radiation on micro-organisms, and it is this part which is sensitive to the growth conditions after irradiation. It is convenient to postulate that radiation inflicts damage at different sites in the cell, so that the total cell death may be attributable to a variety of mechanisms. It has been shown elsewhere that the ratios between surviving fractions on different media were considerably higher after u.v.-irradiation than after ionizing radiation, and, with the latter, the ratios were higher when the irradiations were performed in anoxic than in aerobic conditions (Alper & Gillies, 1957). It is clear that when u.v.-radiation is used, a large proportion of cell deaths may arise from the particular type of damage which leads to unbalanced growth, but this type may not become manifest when post-irradiation conditions are such as to inhibit the unbalanced growth. With ionizing radiations used in aerobic conditions, however, a considerably larger proportion of deaths is due to the absorption of energy in other sites. This latter proportion will appear larger still if after ionizing radiation the conditions are such that the development of unbalanced growth is inhibited. In general, the particular set of experimental conditions used in any irradiation experiment will determine which mechanism of cell death appears to play the most important role.

We are grateful for gifts of strains from Dr G. E. Stapleton (*Escherichia coli* strain B/r); Dr W. Hayes (*E. coli* B and *E. coli* K12/W1485); Dr R. Clowes (*Salmonella typhimurium* type LT2). We are greatly indebted to Mr D. Moore for his co-operation in carrying out the irradiations and dosimetry with the Van de Graaff generator.

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BACTERIAL LYSIS

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Lysis by Physical and Chemical Methods

By B. A. PETHICA

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The term lysis as applied to bacteria may be taken as referring to one or more processes; it has been used to describe the optical clearing of a bacterial suspension (Salton, 1957), and also to describe leakage of small molecular weight materials without optical clearing. The first usage is probably more rigorous and is analogous to the use of the term in haemolysis. For the present communication, however, it is proposed to use the term to cover leakage as well as optical clearing. The term is thus taken to cover what are called prolytic phenomena (e.g. K^+ leakage) in erythrocytes.

To separate for discussion lysis due to 'chemical' or 'physical' agents is also to some extent arbitrary. One may presume that even bacteriophages operate by the laws of physics and chemistry, but it is convenient to focus attention on more or less non-specific methods of lysis. Under physical methods of lysis, one may include heating, freezing, osmosis and shaking, etc., and under chemical lysis the effect of acids and bases, detergents, and the almost non-specific reagents such as alcohols, ether, etc. It is hardly necessary to point out that a physical process such as freezing or a chemical reaction involving detergents, for example, may in fact lead to lysis by initiating a biochemical process such as enzymic autolysis, and that in particular cases no clear distinction between biochemical, physical or chemical lysis is always possible. The present discussion will not consider physical lysis in any detail beyond pointing out certain obvious explanations such as phase changes in membranes on cooling, heat denaturation of proteins, and mechanical fragility on shaking.

In discussing chemical lysis, attention will be focused on the lytic effects of ionic detergents and of non-ionic soluble molecules such as butanol.

The fact that a very large amount of work has been carried out on the lysis of red blood cells makes it convenient to compare and contrast the lytic reactions of bacteria and red cells. In the first place it is useful to compare the structure and activities of the red cell and bacteria. The most obvious difference between the two classes of cell is the absence of a cell wall around the erythrocyte. Functionally the cell wall protects bacteria against osmotic fluctuations not met by erythrocytes. Mitchell (1954) and Mitchell & Moyle (1956*a*) have proved that the protoplasmic membrane of bacteria acts as an osmotic barrier, and that about 70 % of the intracellular water is osmotically available. Weibull (1956) and McQuillen (1956) have shown that bacterial protoplasts act as osmometers and may be lysed by shaking or other means to produce a ghost which can be identified with the protoplast membrane. The protoplast membrane may be identified with the small-particle fraction of disrupted cells and contains about 40 % protein and 20 % lipid in the case of *Staphylococcus aureus* (Mitchell & Moyle, 1956*a*). The lipid of the protoplast membrane is likely to be predominantly phospholipid. The same conclusion also applies to *Micrococcus lysodeikticus* (Gilby & Few, 1957). The exchange of phosphate ions across the membrane is of the exchange diffusion type in resting cells of *Escherichia coli* and *S. aureus*, with little net transport (Mitchell & Moyle, 1956*b*). These properties, and the lack of mechanical rigidity of the protoplast membrane (Mitchell & Moyle, 1956*a*) show great similarities between bacterial protoplasts and red cells (Ponder, 1948). On the other hand, the protoplast membrane is the site of many enzymes, whereas only acetylcholine esterase has been definitely located in the red cell stroma (Paléus, 1947). In addition, the overall lytic properties of bacteria depend on the growth phase, the strength of the cell wall, degree of metabolic activity, aerobiosis, etc. Mitchell & Moyle (1956*b*) have given strong thermodynamic reasons for regarding the protoplast membrane as an ordered lipoprotein layered structure similar to that proposed by Stein & Danielli (1956), which is permeable to phosphate by a process of reversible denaturation. The red cell membrane may also be regarded in this way (Adair, 1956). Electrophoretic data shows that the erythrocyte exterior surface is probably lipid, with a predominance of charged phosphate groups (Seaman & Pethica, 1957), whereas the protoplast of *M. lysodeikticus* has an exterior surface of a protein-type (Seaman, G. V. F., unpublished results, 1957). This difference may be related to the adhesion of the protoplast to the cell wall. Further differences between erythrocytes and bacterial protoplast membranes are that no cholesterol is found in bacterial membranes (Gilby & Few, 1957), and that whereas erythrocytes are more permeable to anions than cations, the reverse is likely to be true for some bacteria, such as *S. aureus* (Mitchell & Moyle, 1956*b*).

This brief account of the comparative properties of red cell and bacterial membranes enables certain conclusions to be drawn about the mechanism of the lytic action of detergents on bacteria. There is abundant evidence that the ionic detergents are bactericidal through their effect on the cytoplasmic membrane (Schulman, Pethica, Few & Salton, 1955; Salton, 1957). The leakage of small molecules (e.g. nucleotides) induced by ionic detergents is in general more easily brought about with Gram-positive than with Gram-negative bacteria. This difference is related to differences in the cell walls of Gram-positive and Gram-negative bacteria. The Gram-negative organisms so far studied have a high lipid content in the cell walls, whereas the cell walls of Gram-positive organisms have a low lipid content (Salton, 1952, 1953). Salton (1957) suggested that the screening effect of the cell-wall lipids, which will react with detergents, protects the cytoplasmic membrane of Gram-negative organisms. The effect of detergents in dispersing the cell walls themselves, and thus permitting optical clearing of the bacterial suspension, has also been discussed by Salton (1957). After the initial rapid effect of the detergents in causing leakage of small molecules, a slower process which terminates in the complete lysis of the cells is often observed. This process is ascribed to the action of autolytic enzymes on the disorganized or dead cell (Salton, 1951; Dawson, Lominski & Stern, 1953). In the case of the Gram-negative *Proteus vulgaris* and *Pseudomonas viscosa* the full lysis effected by sodium dodecyl sulphate and Teepol is probably due to a direct action of the detergent on the lipid-rich cell wall (Salton, 1957; Lominski & Lendrum, 1942). The detergent in this case will act strictly as a detergent, 'cleaning' the membrane of the lipids. Under these circumstances the cytoplasmic membrane is also destroyed leaving no ghost (Salton, 1957). This destruction of the ghost is also found when dodecyl sulphate lyses erythrocytes (Pethica, B. A., unpublished results, 1957). The ionic detergents when not acting directly to dissolve the wall, may increase the rate of autolysis by removal of inhibitors of autolysis. It may also be noted that detergents affect enzymes markedly. Sodium dodecyl sulphate, for example, inhibits succinic oxidase, urease, pancreatic lipase, invertase, papain and amylase, but stimulates the action of trypsin on native proteins (Wills, 1954). Sodium dodecyl sulphate also inhibits acetycholine esterase (Anderson, 1954), but has little effect on serum choline esterase or cytochrome oxidase (Wills, 1954). Bacterial walls contain few, if any, enzymes (Weibull, 1956). Detergents acting on the enzyme-rich cytoplasmic membrane could thus easily allow contact and reaction between the wall and enzymes which normally are kept structurally away from the wall.

The actual site of reaction of the ionic detergents on the protoplast membrane is not definitely known. Detergents are known to denature proteins (Putnam, 1948), inhibit enzymes (Wills, 1954) and interact with lipids at interfaces (Schulman & Rideal, 1937; Pethica, 1955). The haemolytic effect of a range of ionic detergents is parallel to their ability to penetrate monolayers of cholesterol (Pethica & Schulman, 1953; Schulman *et al.* 1955; Pethica & Anderson, 1953). Cholesterol is thus a likely primary site of attack for ionic detergents acting on red cells. Gilby & Few (1957) showed with the dodecyl series of ionic detergents, that the lytic action against the protoplast of *Micrococcus lysodeikticus* was in the order of head groups:

$$C_{12}H_{25}NH_3^+ > NMe_3^+ > SO_4^- > SO_3^-.$$

This sequence is contrary to the haemolytic series. Anderson & Pethica (1955) showed that dodecylamine interacts more effectively than dodecyl sulphate with lecithin monolayers, which is in the correct order for protoplast lysis. Until more evidence is forthcoming it may be tentatively assumed that the ionic detergents interact principally with phospholipids of the bacterial membrane. This suggestion is reinforced by the fact that dodecyl trimethyl ammonium bromide interacts poorly with the protein bovine plasma albumin, whereas sodium dodecyl sulphate interacts strongly with this and other proteins (Few, Ottewill & Parreira, 1955; Putnam, 1948). Physico-chemically the effect of these detergents is to break down irreversibly a lipoprotein structure either by removal (detergency) of important components of the membrane or by disordering the structure by penetration.

The ionic detergents may be expected to engage in fairly specific reactions with membrane components because of the high energies of ion-dipole and ion-ion interactions of detergents (Pethica, 1955; Goddard & Schulman, 1953). In the case of non-ionic surface active agents this specificity will be much lower. It was shown for haemolysis that non-ionic surface active agents of various types cause lysis when the surface pressure of the solution (lowering of the air/solution surface tension) was >34 dynes/cm. For butanol the concentration at which this surface pressure is attained is 0·39 M. Mitchell (1953) showed that leakage of phosphate occurred from *Staphylococcus aureus* above a critical concentration of 0·4 M. Dr Mitchell has now shown that the same critical concentration of butanol causes leakage in *Micrococcus lysodeikticus*, *Sarcina lutea* and *Escherichia coli*. It is also known that irreversible blocking of the frog sciatic nerve occurs at a butanol concentration above 0·4 M (Skou, 1954). A similar concentration of butanol breaks down the ram spermatozoon membrane (Mann, T., private communication). Kaplan (1954) showed that yeast catalase was removed from an intracellular surface structure by butanol at 0·33 M and by a range of aldehydes, ketones and alcohols giving a surface tension lowering of about 32 dynes/cm. These effects in a range of biological systems suggest that a common membrane link or structure is being altered by butanol and similar agents (Pethica, 1956). It was suggested for red cells that the critical surface pressure for lysis indicated a maximum two-dimensional free energy beyond which the surface became occupied by the non-ionic surface active agent with collapse of the membrane (Pethica & Schulman, 1953). Other explanations of the effect of alcohols are possible. Stein & Danielli (1956) suggested that butanol can act as a hydrogen-bonding agent in disrupting the hydrogen-bonded structure of the membrane of a red cell. The similarity of the action of butanol in this range of lytic and irreversible blocking phenomena calls to mind the work of Morton (1953, 1954), who showed that butanol was able to release an alkaline phosphatase from association with a lipoprotein in milk and calf intestinal mucosa. Regarding the membrane as a lipoprotein complex, the effect of butanol and similar non-ionic surface active agents may be ascribed to breaking of lipid-protein (enzyme) linkages, either by acting as a hydrogen-bond breaker or by competing for the ionic components of ion-dipole linkages. The effect of butanol and similar compounds in narcosis may be regarded as an early, reversible stage of membrane breakdown. Narcosis is essentially reversible and occurs at lower concentrations than lysis or irreversible alteration of membranes. The question as to whether narcotics act on membranes or on intracellular lipids has often been debated (Dethier, 1954). In the case of the red cell, the lipids of the cell are concentrated in the membrane (Ponder, 1948), and butanol and other narcotic agents may be presumed to act at the membrane. Since excitability of the nerve cell is related to the ion permeability of the nerve membrane, the narcotic and blocking action of butanol, etc., will also be at the membrane surface. Accepting the view that the permeability of biological membranes is a process involving a reversible denaturation of the membrane structure (Mitchell, 1954; Adair, 1956), the effect of low concentrations of butanol and other narcotics will be to reversibly modify the membrane structure to allow ion leakage. In red cells this is manifest as an exchange of K^+ and Na^+ without lysis, at sublytic butanol concentrations. In the nerve cell, the alteration of ion permeability at narcotic concentrations causes loss of excitability. It would be interesting to observe whether ion leakage of K^+ occurs at narcotic concentrations with bacteria. At higher concentrations, the alteration of the membrane becomes critical leading to haemolysis (leakage of haemoglobin), irreversible blockage of nerve cells, breakdown of the specific phosphate exchange mechanism in bacteria and leakage of nucleotides, etc. It is likely that the effect of pH and other factors (e.g. sucrose concentration) on the critical lytic concentrations of butanol will give valuable information on cell-membrane bonding.

In concluding this contribution to the discussion it would seem permissible for a physical chemist to make a few remarks on the interpretation of a number of experimental variables which are important in studies of bacterial and other lysis. The variables in question are pH value, salt concentration and temperature. These three variables should be regarded as interdependent. The importance of the pH value needs no emphasis, but it is often overlooked that measurements of pH carried out in the conventional manner are not sufficient in themselves to define the effective concentration of hydrogen ions at biological membranes. At any surface which carries ionic charges the effective concentration of hydrogen ions near the surface is a function of the pH value of the bulk of the solution (as measured on a pH meter) and of the surface potential. Assuming a Boltzmann distribution and no solvation effects, the pH value near a surface (pH_s) is given by:

$$\mathrm{pH}_s = \mathrm{pH} + \frac{e\psi}{2{\cdot}3kT}, \tag{1}$$

where ψ is the surface or double layer potential, e the proton charge, k the Boltzmann constant and T the absolute temperature (Danielli, 1937; Hartley & Roe, 1940; Betts & Pethica, 1956). The value of ψ depends on the surface-charge density and the salt concentration for a given pH value. The term 'salt concentration' is used in preference to 'ionic strength' here because ionic strength is a property of a salt solution relevant to ionic concentrations in three dimensions. Near a surface, the most important concentration in defining the colloid properties of the surface and the value of ψ is the concentration of ions of opposite sign to the surface charge. To keep ionic strength constant is to treat the system as three dimensional. For a surface system the salt concentration should be adjusted to keep the counter ions at an effective constant concentration. This may be calculated in the absence of specific binding from the Gouy theory (Verwey & Overbeek, 1948). To give an example, 0·1 M-NaCl is of equal ionic strength with 0·033 M-Na_2SO_4, but has an equivalent Gouy concentration of 0·025 M for a positive surface and 0·05 M for a negative surface at low-charge densities. Variations of bulk pH value which are accompanied by changes in salt concentration as discussed above, thus alter the effective pH values at membranes, etc., as a function of two variables, and not of one. The importances of differences in pH values between biological surfaces and bulk solutions may be realized from the fact that for a close-packed fatty acid monolayer, only a small degree of ionization takes place at pH = 4·8 (the nominal half-ionization pH value) in 0·1 M-NaCl. Dilution of the NaCl at constant pH depresses the ionization in the surface still further (Betts & Pethica, 1956). The importance of surface pH has been discussed by Frazer (1957) and by McLaren (1957) in relation to enzyme reactions at interfaces. The relevance of surface pH value to oxidation-reduction reactions involving charged thiol compounds is discussed by Danielli & Davies (1951). Surface pH has been taken into account in interpreting the ionization of charged groups at the surface of human erythrocytes (Seaman & Pethica, 1957) and of *Escherichia coli* (Davies, Haydon & Rideal, 1956).

The effect of temperature on biological reactions is often interpreted in terms of activation energies. It is well known that activation energies for enzyme reactions may include the heat of ionization of the reactive group on the enzyme when this is weakly ionized, due to the change of pK of the active groups with temperature (Gutfreund, 1955). This same consideration also applies to reactions at charged membranes. In addition, a membrane charge which is not directly concerned in a reaction will also influence the rate of a reaction if the reagent is charged. Thus activation energies for charged molecules permeating a membrane will be related to the intrinsic activation of the specific permeation reaction and to the change of potential at the membrane resulting from the change of membrane charge with temperature. An increased negative membrane-potential will decrease the apparent activation energy of a positive molecule and increase the apparent activation for a negative molecule. We may note that Mitchell's (1954) calculation of the activation energy for phosphate transfer in *Staphylococcus aureus* may be in error for this reason, although the probable magnitude of the error is insufficient to alter his interpretation of the activation energy. Once again the importance of salt concentration should be borne in mind, in that the activation energies for charged molecules will also depend, in general, on the effect of salts on the surface potential of the membrane.

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The Lysis of Micro-organisms by Lysozyme and Related Enzymes

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'Lysozyme' was the name given by Fleming (1922) to an enzyme, present in various tissues and secretions, which possessed the ability to lyse the cells of many microbial species. Of all the sources tested for 'lysozyme' activity, Fleming (1922) found that egg white contained the highest amounts. Because of the abundance of egg white as a source of enzyme, the action of egg-white lysozyme on micro-organisms has been investigated more fully than 'lysozymes' from other tissues. Alderton, Ward & Fevold (1945) succeeded in isolating lysozyme from egg white in the form of a crystalline protein, and in sufficient yield to determine its homogeneity and physico-chemical properties. Enzymes possessing lytic activities in common with egg-white lysozyme have been isolated as crystalline proteins or protein derivatives from rabbit spleen (Jollès & Fromageot, 1954) and from papaya latex (Smith, Kimmel, Brown & Thompson, 1955). The lysozymes thus obtained from egg white, rabbit spleen and papaya latex, have several features in common. They are basic proteins of comparatively low molecular weights (*c.* 14,000 for egg-white and rabbit-spleen enzymes and *c.* 24,000 for papaya-latex enzyme, Fevold, 1951; Jollès & Fromageot, 1954; Smith *et al.* 1955) are devoid of sulphydryl groups and have rather similar numbers of residues of certain amino acids. Egg-white lysozyme and the rabbit-spleen enzyme have lysine as the *N*-terminal amino acid and leucine as the *C*-terminal amino acid (data recently summarized by Salton, 1957*a*).

Apart from the similarities in the range of micro-organisms lysed, there is no detailed information about the nature of the bonds broken by the enzymes from rabbit spleen and papaya latex. The lytic properties of purified enzymes related to egg-white lysozyme have been reported by a number of investigators, e.g. the enzyme from polymorphonuclear leucocytes (Epstein & Chain, 1940); plakin (Amano, Kato & Shimizu, 1952); bacterial lysozyme from *Bacillus subtilis* (Richmond, 1957). The bacterial enzyme described by Richmond (1957) appears to be very similar to egg-white lysozyme, especially in respect of the nature of the non-dialysable products obtained by its action on cell-wall substrates. Although many of the above-mentioned enzymes are closely related to egg-white lysozyme and may eventually be shown to act on similar substrate groups, the subsequent use of the term 'lysozyme' in this paper, will apply only to egg-white lysozyme, for it has been this enzyme that has been used in determining the manner in which lysozyme acts upon micro-organisms.

The action of lysozyme on Gram-positive bacteria

The lytic action of lysozyme on bacteria can be ascribed simply to the dissolution of the rigid cell-wall structures. When the degradation of the wall occurs in dilute media, the underlying structures of a lysozyme-sensitive bacterial cell will collapse and the liberation of the cytoplasmic components into the medium will result in the lysis of the bacterial suspension. However, Weibull (1953) showed that dissolution of the wall of *Bacillus megaterium* in the presence of osmotically protective quantities of sucrose was accompanied by liberation of bacterial protoplasts and only partial optical clearing of the cell suspension. Although protoplast formation may account for incomplete lysis of bacterial suspensions when treated with lysozyme under appropriate conditions, it is evident that the walls of bacteria differ quantitatively in the 'amount' of lysozyme substrate present and such a factor as this may contribute to the partial optical clearing in dilute media.

For a detailed investigation of the mechanism of lysozyme action, three Gram-positive bacteria whose isolated walls were completely digested by lysozyme were selected (Salton, 1956*a*). On incubation with crystalline egg-white lysozyme, the walls of *Micrococcus lysodeikticus*, *Sarcina lutea* and *Bacillus megaterium* are digested with a liberation of reducing and acetyl amino sugar groups. Lysozyme activity may be followed by measuring the decrease in the turbidity of the cell-wall suspensions and by estimating the amounts of liberated reducing substances. With cell walls of the above-mentioned organisms, the optimum pH value for lysozyme activity as determined by the decrease in turbidity or liberation of reducing substances, is between pH 6·5 and 7·0. The optimum pH value for the liberation of reducing groups is sensitive to variations in the ionic strengths of the buffer systems, discontinuities appearing in the pH curves when the ionic strengths are not controlled at constant values. The dissolution of the walls of *M. lysodeikticus* by lysozyme shows a broad optimum ionic strength requirement of $\mu = 0{\cdot}1$ (Salton & Ghuysen, 1957). This is in marked contrast to the very narrow ionic strength optima observed by Ghuysen (1957) for the action of his purified enzymes from *Streptomyces albus* on certain intact bacteria and cell-wall substrates.

The products resulting from the digestion of the walls of *Micrococcus lysodeikticus*, *Sarcina lutea* and *Bacillus megaterium* are complex mixtures of fragments of differing molecular sizes (the largest fragments have molecular weights of the order of 10,000–20,000) and electrophoretic properties (Salton, 1956*a*). The larger non-dialysable fragments and the dialysable fractions give acetyl amino sugar reactions. The main dialysable substance liberated by lysozyme appears to be a disaccharide of acetylglucosamine and acetyl 'muramic acid' ('muramic acid' is the name Strange has suggested for an acidic amino sugar isolated from bacterial cell walls and spore peptides by Strange & Dark, 1956; Strange (1956) has provisionally proposed the structure of 'muramic acid' as 3-*O*-α-carboxyethyl hexosamine). The cell-wall amino acids and sugars are not detectable as the free substances in the dialysable fractions of the digests. The available evidence thus suggests that lysozyme is splitting the glycosidic bonds of the cell-wall amino sugars, liberating the disaccharide of acetylglucosamine and acetyl 'muramic acid' as the simplest product, together with more complex fragments which differ quantitatively rather than qualitatively in their chemical composition. It is not known at present, whether the cell-wall amino sugars are linked by α- or β-glycosidic bonds. That the main action of lysozyme is one involving amino sugar bonds is further strengthened by the observation that it also attacks cell walls with their free amino groups blocked by reaction with 1-fluoro-2:4-dinitrobenzene (FDNB) at about the same rate as the untreated walls (Ingram & Salton, 1957). There is, however, an increase in the number of *N*-terminal alanine molecules after the walls have been digested with lysozyme. If this is not due merely to the more complete reaction of the soluble digested walls with FDNB, as compared with insoluble walls, then it must be assumed that there is a subsidiary action of lysozyme on other parts of the cell-wall structure. However, the increase in terminal alanine groups after digestion is small in comparison with the liberation of reducing groups (Ingram & Salton, 1957).

In more recent investigations I have been endeavouring to find out whether lysozyme sensitivity is correlated with any special features of the composition of the cell walls—in effect to see whether one could 'diagnose' the lysozyme substrate from the quality and/or quantity of cell-wall constituents. From the many lytic tests reported for the sensitivity of intact cells of a large number of micro-organisms, it seemed likely that a whole 'spectrum of sensitivities' to lysozyme might be expected when tests were performed on isolated cell walls. This has indeed proved to be so and the results of the dissolution of the walls of various staphylococci and micrococci by lysozyme, presented in Fig. 1. illustrate such a range of susceptibilities to digestion. The final degrees of decrease in turbidity after 24 hr.-incubation with lysozyme, varied from 32 % for *Staphylococcus citreus* to 80 % for *Micrococcus roseus* and 98 % for *Sporosarcina ureae*. It seems probable that a similar 'spectrum of sensitivities' exists for members of the genus *Bacillus*; *B. megaterium* cell walls are completely digested by lysozyme, whereas walls of *B. subtilis* and *B. cereus* are only partially dissolved (*c.* 50 % turbidity decrease in both instances) and the rate of dissolution of the walls of the latter organism is much lower than that observed for the other species (Salton, 1956*b*).

The principal amino acid, amino sugar and sugar constituents of the various cell walls showing different sensitivities to lysozyme, have been determined by paper chromatographic examinations of hydrolysed walls (6 N-HCl for amino acids; 2 N-H_2SO_4 for sugars; amino sugars detectable under both conditions of acid hydrolysis). The major amino acid, amino sugar and sugar constituents found in the walls are summarized in Table 1 and data on the lysozyme sensitivity and contents of amino sugar are presented in Table 2. These results, in common with the more extensive surveys reported by Cummins & Harris (1956*a*, *b*), have shown that alanine, glutamic acid, glucosamine and 'muramic acid' are present in the cell walls of all of the Gram-positive bacteria so far investigated. Inspection of the results given in Tables 1 and 2 shows that there is no qualitative or quantitative correlation between cell-wall constituents and sensitivity to lysozyme. Thus both 'lysozyme-sensitive' and 'lysozyme-resistant' bacterial cell walls contain alanine, glutamic acid and the two amino sugars. A comparison of the amino sugar contents of the resistant walls of *Streptococcus faecalis* (22 %) and the partially-sensitive walls of *Bacillus cereus* (31 % amino sugars), with those of the susceptible *Micrococcus lysodeikticus* (16–18 %) and *B. megaterium* (18 %), shows no relationship between this factor and lysozyme susceptibility. It is therefore clear that at the present moment we cannot predict the lysozyme sensitivity of the wall of a particular bacterial species by simple chromatographic identification of its constituents or by estimation of the amino sugar contents. Such a situation is perhaps not surprising since mere identification and even estimation of cell-wall constituents tells us nothing of the arrangement of the amino acid, amino sugar or sugar molecules in the polymerized cell-wall structure. The presence

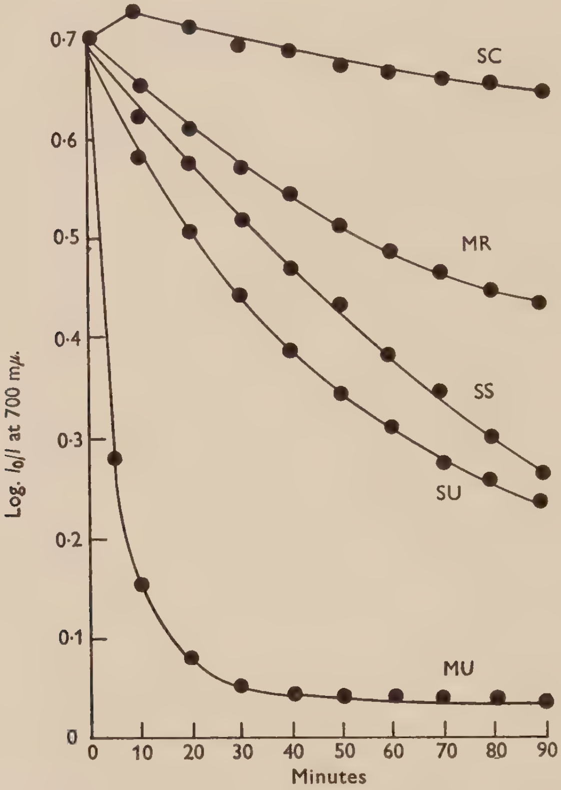

Fig. 1. The action of lysozyme on the cell walls of SC (*Staphylococcus citreus*), MR (*Micrococcus roseus*), SS (*Staphylococcus saprophyticus*), SU (*Sporosarcina ureae*), MU (*Micrococcus urea*). Cell walls treated with lysozyme (50 μg./ml. final concentration; pH 7; 37°), and turbidity decrease followed by measurement in the Unicam spectrophotometer, model SP 600.

of galactosamine in the walls of *S. faecalis* and those of *Clostridium welchii* may be correlated with their resistance, but until more species containing all three amino sugars in their walls have been examined for lysozyme sensitivity, such a correlation must remain a superficial one. Thus it seems improbable that we shall be able to explain the factors responsible for the susceptibility of some bacterial walls to lysozyme until we know more about the frequency with which the disaccharide occurs in the wall and the way in which the amino sugars are linked to one another and to other cell-wall constituents.

Table 1. *Amino acid, amino sugar and sugar constituents of cell walls of Gram-negative bacteria*

Cell walls (arranged in order of decreasing sensitivity to lysozyme)	Amino acids					Amino sugars			Sugars		
	Al	Glu	Ly	Gly	DAP	Gluca	Gala	Mur	Gluc	Gal	Rham
Micrococcus lysodeikticus	+	+	+	+	−	+	−	+	+	−	−
Sarcina lutea	+	+	+	+	−	+	−	+	+	−	−
Sporosarcina ureae	+	+	+	+	−	+	−	+	−	−	−
Staphylococcus saprophyticus	+	+	+	+	−	+	−	+	+	tr	−
Micrococcus varians	+	+	−	+	+	+	−	+	+	−	−
Bacillus megaterium	+	+	−	−	+	+	−	+	−	+	−
Micrococcus tetragenus	+	+	+	+	−	+	−	+	+	tr	−
M. roseus	+	+	+	−	−	+	−	+	−	+	−
Bacillus subtilis	+	+	−	−	+	+	−	+	+	−	−
B. cereus	+	+	−	−	+	+	−	+	+	−	−
Staphylococcus citreus	+	+	+	+	−	+	−	+	+	−	−
Streptococcus faecalis	+	+	+	+	−	+	+	+	+	+	+
Clostridium welchii	+	+	−	+	+	+	+	+	+	−	+

Abbreviations: Amino acids: Al, alanine; Glu, glutamic acid; Ly, lysine; Gly, glycine; DAP, diaminopimelic acid. Amino sugars: Gluca, glucosamine; Gala, galactosamine; Mur, 'muramic acid'. Sugars: Gluc, glucose; Gal, galactose; Rham, rhamnose.

N.B. No attempt has been made to indicate the relative proportions of cell-wall constituents.

Table 2. *Lysozyme sensitivity and amino sugar contents of cell walls of Gram-negative bacteria*

Cell walls from	Order of decreasing sensitivity to lysozyme (% turbidity decrease)	% amino sugar (2 N-HCl hydrolysates)
Micrococcus lysodeikticus	> 90	16–18
Sarcina lutea	> 90	14–16
Sporosarcina ureae	> 90	14
Staphylococcus saprophyticus	> 90	14
Micrococcus varians	> 90	10
Bacillus megaterium	> 90	18
Micrococcus tetragenus	50–90	13
M. roseus	50–90	15
Bacillus subtilis	50	8·5
B. cereus	50	31
Staphylococcus citreus	< 50	10
Streptococcus faecalis	< 10	22
Clostridium welchii	< 10	—

The action of lysozyme on Gram-negative bacteria

As a group the Gram-negative bacteria have fewer members as sensitive to lysozyme as some of the Gram-positive species from the genus *Micrococcus* or from the genus *Bacillus*. However, it is evident that some Gram-negative bacteria are at least as susceptible to lysis by lysozyme as the more resistant species of Gram-positive bacteria. Thus *Brucella abortus* is lysed by lysozyme and appreciable quantities of the substrate can be extracted from the cells (Epstein & Chain, 1940). Unlike the Gram-positive bacteria, the lysozyme sensitivity of intact cells of many Gram-negative organisms can often be established only after the application of special techniques such as the following: (1) the addition of sodium hydroxide solutions after exposure to lysozyme under the conditions described by Nakamura (1923); (2) incubation with lysozyme at low pH values (*c.* 3·5) at 45° followed by the addition of alkali *c.* pH 10 (Peterson & Hartsell, 1955; Grula & Hartsell, 1957*a*, *b*); (3) prior treatment of bacteria with acetone or heat (Warren, Gray & Bartell, 1955); (4) treatment with lysozyme in the presence of 'Versene' (ethylenediamine tetra-acetic acid—EDTA) as reported by Repaske (1956). Thus with many Gram-negative bacteria, the lysis due to lysozyme is often negligible, but the contribution due to lysozyme when the treatment is combined with any of the above procedures, is frequently quite large. Grula & Hartsell (1957*a*, *b*) determined the % changes in optical densities of bacterial suspensions due to the addition of alkali and due to the combined action of lysozyme and subsequent treatment with alkali. The results showed that lysozyme contributed to a greater or lesser degree to the optical clearing of suspensions of all organisms except *Brucella suis*. This work again indicates that the differences in susceptibility to the lytic action of lysozyme are quantitative rather than qualitative.

Because the chemical constitution of the cell walls of Gram-negative bacteria differ in a number of respects from those of Gram-positive organisms (Salton, 1953; 1957*b*), the question of their sensitivity to lysozyme has become an intriguing problem. The major components of the walls of many Gram-negative bacteria are proteins, lipids and polysaccharides. In contrast to this, the walls of Gram-positive bacteria are of muco-complex nature, containing peptides and amino sugar and sugar constituents with little or no lipid (Salton, 1953). The walls of all of the Gram-negative bacteria so far examined, contain glucosamine, 'muramic acid' and diaminopimelic acid (DAP) (Salton, 1957*b*). Thus the two amino sugars are common to cell walls from organisms of both Gram-positive and Gram-negative groups. As shown in Table 3, the amino sugar contents of the cell walls of Gram-negative bacteria are generally lower than those of walls from Gram-positive organisms.

When the isolated cell walls of certain Gram-negative bacteria are incubated with lysozyme under conditions giving complete digestion of the walls of a sensitive organism such as *Micrococcus lysodeikticus*, only a small turbidity decrease can be detected. For example, the walls of a strain of *Escherichia coli* showed a maximum turbidity decrease of 4 % on incubation with lysozyme

(100 μg. crystalline egg-white lysozyme/ml.; pH 7; ionic strength $\mu = 0{\cdot}1$; 37°; 24 hr.) and the corresponding value for walls of *Salmonella gallinarum* was 19 %. However, despite the small changes in turbidity, an examination of the insoluble and soluble fractions of walls from *E. coli*, *S. gallinarum*, a psychrophilic strain of *Pseudomonas* sp. and *Chlorobium thiosulphatophilum* after incubation with lysozyme, has given some indication of the nature of lysozyme action on these structures.

Table 3. *Amino sugar content of the cell walls of Gram-negative bacteria*

Cell walls from	% amino sugar (2N-HCl hydrolysates)
Escherichia coli	2·2–4·0
Spirillum serpens	6·8
Chlorobium thiosulphatophilum	4·2
Rhodospirillum rubrum	2
Vibrio metchnikovi	1·9
Salmonella pullorum	4·8
S. gallinarum	3·9
Organism LC 1*	13

* A Gram-negative coccus described by Elsden, Volcani, Gilchrist & Lewis (1956).

Table 4. *Amino sugar contents of cell walls and lysozyme-insoluble wall fractions of two Gram-negative bacteria*

	Original cell wall	Lysozyme-insoluble residues
	% amino sugars (2N-HCl hydrolysates)	
Chlorobium thiosulphatophilum	4·2	0·7
Salmonella gallinarum	3·9	1·8

The soluble, non-dialysable components released by lysozyme from the walls of the above Gram-negative bacteria, contained alanine, glutamic acid, DAP and glucosamine as the predominant substances, with smaller amounts of 'muramic acid' and a number of other amino acids. The most conspicuous features of the paper chromatograms of the hydrolysed, lysozyme-insoluble residues, were the presence of all of the original cell-wall amino acids, with the exception of DAP which was detectable only in trace amounts, the presence of glucosamine, and only faint traces of 'muramic acid'. A comparison of paper chromatograms from the hydrolysed insoluble residues with those from the hydrolysed soluble, non-dialysable fractions, also showed that all but a trace of the cell-wall tyrosine was located in the lysozyme-insoluble wall fraction. The changes in amino sugar components indicated by paper chromatography have been confirmed for several preparations by determining the amino sugar contents of the original preparations and the insoluble residues after lysozyme action. These data are given in Table 4.

Thus lysozyme action on the cell walls of the Gram-negative bacteria, used in this investigation, deprives the residual cell-wall structures of most of their DAP and 'muramic acid', and releases a soluble component in which alanine, glutamic acid and DAP are the principal amino acid constituents. Such a product composed of glucosamine, 'muramic acid' and a limited range of amino acids is obviously related to the class of substances forming the entire framework of the cell walls of many Gram-positive bacteria. These results, considered in the light of the effects of the Nakamura technique (Grula & Hartsell, 1957*a*, *b*), EDTA (Repaske, 1956) and disaggregating agents such as sodium dodecyl sulphate (Salton, 1957*c*), give us a clearer picture of the architecture of the walls of some Gram-negative bacteria. The evidence so far available suggests that the walls of Gram-negative bacteria are composed of two main structural entities: (1) a rigid muco-complex component of the type forming the entire wall of many Gram-positive bacteria; this component may form the rigid 'backbone' of the cell wall and be responsible for the characteristic shape of a particular Gram-negative species; (2) a structurally less rigid lipoprotein component, held together by weaker bonds that would be susceptible to reagents (e.g. EDTA, sodium dodecyl sulphate) capable of disaggregating lipoprotein complexes. The relative proportions of the two components in the walls probably vary from one species to another.

The dual features of the cell-wall structure of Gram-negative bacteria suggested above is compatible with observations of the loss of cell-wall rigidity and of 'protoplast' formation by lysozyme treatment of *Escherichia coli* at high pH values (Zinder & Arndt, 1956) or with 'Versene' (Mahler & Fraser, 1956) and the production of 'protoplasts' of *Escherichia coli* on media containing penicillin (Lederberg, 1956). Recent experiments performed by Dr F. Shafa and myself have indicated that both lysozyme action and penicillin action probably involve the same type of structure in the cell walls of certain Gram-negative bacteria. The two organisms *Vibrio metchnikovi* and *Salmonella gallinarum* are readily transformed to spherical forms (so-called 'protoplasts') on media containing penicillin, and this transformation is accompanied by a loss of cell-wall amino sugar and DAP. Although the cell walls of these organisms are not destroyed during this spherical transformation (or 'protoplast' formation) on penicillin agar media, it is evident that the synthesis of the components essential for the rigidity of the cell-wall structure blocked by penicillin and the pre-existing muco-complex components of the wall are probably then 'diluted out'. Thus any treatment of Gram-negative micro-organisms effecting a loss of cell-wall muco-complex, either by direct enzymic degradation as with lysozyme, or by blocked synthesis as with penicillin, will result in a more plastic cell-wall structure of the lipoprotein type.

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Lysis by Agents of Microbial Origin

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Instances of bacterial cultures undergoing spontaneous dissolution, and examples of micro-organisms or microbial products digesting the cells of various bacteria have often been reported since the very beginnings of bacteriology. Research in this field was undertaken from many different angles according to the fashion of the day. Early studies were mostly conducted with practical applications in view. Thus lysates were studied as vaccines. Later on, it was announced by Schiller that highly specific bacterial lysins could be obtained, almost at will, through a process called induced or forced antagonism, and that such lysins had therapeutic virtues. This idea emerged again and again during the ensuing years, but was never satisfactorily proved. Bacteriolysis was also used as a tool in order to obtain, free from cells, components with antigenic, toxic, enzymic or other properties.

The biological significance of bacteriolysis did not escape attention. Its role in Nature, as a factor involved in the cycle of the biogenic elements, was pointed out early by A. Gratia and later on by others. Hints can also be found in the literature of a possible relation between autolysis, on the one hand, and spore formation and germination on the other hand (Halvorson & Church, 1957), as well as between autolysis and the release of exotoxins (Boroff, 1955). The importance of the lytic process in bacteriophage infection has long been recognized, but its study was rather overshadowed by that of the bacterial virus itself. Lastly, the mechanism of bacteriolysis attracted the attention of scientists. In this connexion, the possible role of the Gram-positive substrate was first stressed. More recently, and mainly following Salton's work on lysozyme (Salton, 1957), emphasis was laid on the role of the cell wall (Work, 1957).

It thus appears that the subject of the present report is indeed a very broad one. As it would be impossible to cover all its aspects, a few topics will be selected and briefly considered, the reader being referred to a recent review (Welsch, 1957) for more details and additional references. The main purpose of this paper will be to show that a great number of received ideas will have to be revised in the light of modern knowledge of bacterial anatomy.

The most obvious manifestation of bacteriolysis—what indeed should only be called lysis in the strictest sense of the word—is the progressive clearing of an initially turbid bacterial suspension. This may occur without the help of any exogenous agent: it is then called autolysis. On the contrary, it may be promoted by an extraneous agent and can then be described as heterolysis. The exogenous agent may be mechanical, physical, chemical or biological. We are concerned here with the latter case, and only with the particular instance when the agent promoting lysis has a microbial origin. Two different situations are to be considered: either the microbial agent at play, having a truly and directly bacteriolytic action, dissolves the cell or part of it, or it simply modifies the bacteria submitted to its action in such a way that they undergo autolysis. An agent of the latter type can be called an inducer of autolysis. It follows that an examination of the autolytic process is a pre-requisite to the study of heterolysis.

However, it can be no simple matter to decide, in a given instance, whether one deals with a truly bacteriolytic agent or with a mere inducer of autolysis. A valid criterion allowing the choice between these two hypotheses would be the possibility of using a highly specific inhibitor of the autolytic system. Indeed, if such an inhibitor did prevent heterolysis, one might safely conclude that the phenomenon observed was induced autolysis. Unfortunately we have no means of inhibiting autolysis without simultaneously more or less injuring the bacteria. In fact, inhibition of the autolytic system is most often obtained by heating, a treatment which is likely to alter the outer bacterial structures and to thus expose cell substrates which are otherwise protected. Also, heat denaturation can make sensitive to the lytic enzymes parts of the cell that are normally resistant, and conversely. Lastly, cell components are likely to diffuse from the cell during heating. Examples of such situations are known, and the use of heated bacteria has been criticized by Salton. Keeping these important reservations in mind, I believe, nevertheless, that study of the bacteriolysis of killed bacteria can give useful information. In particular, it should be stressed that microbial enzymes able to dissolve heated bacteria (e.g. actinozyme) do not act merely as the usual proteases of the digestive juices but can display a definite specificity for their bacterial substrate.

It is generally taken for granted that autolysis is an enzymic phenomenon ascribable to the action of a number of different enzymes, making, all together, what is often called the *autolytic system*. According to Dubos, the infra-microscopic organization of the living cell prevents the hydrolytic enzymes from digesting the cell components. When, however, the intracellular heterogeneity is not maintained for any reason whatsoever, enzymes and potential substrates are mixed together, the former digesting the latter and autolysis ensuing. Energy being necessary to maintain the cellular infra-structure, it follows that any agent, endogenous or exogenous, which interferes with energy supply or utilization may induce the process of autolysis. Of course, the autolytic systems of different bacteria are more or less potent and stable and this explains their greater or smaller liability to undergo lysis. In keeping with modern observations, it may also be supposed that bacterial species which are particularly resistant to autolysis are so because they lack an enzyme capable of destroying their cell wall and of thus promoting their ultimate disintegration.

If autolysis of a cell is a strictly endogenous process, autolysis of a bacterial population, on the contrary, is both endogenous and exogenous, since the autolytic system liberated from lysed individuals can conceivably act upon other members of the population from the outside. Such is the case, as we have shown, in autolysis of staphylococci. The autolytic system of this organism is released through autolysis and also, in suitable conditions, by growing colonies, provided that a minimal amount of phosphate is present. It dissolves not only the homologous and other strains of staphylococci, but also *Micrococcus lysodeikticus*, *Sarcina lutea* and, to a certain extent, *Escherichia coli* and *Proteus vulgaris*. It is certainly a complex mixture of enzymes, among which a lysozyme-like agent might be present (Mitchell & Moyle, 1957).

When liberated from the cells and used as an agent to promote heterolysis, the autolytic system is frequently called an *autolysin*. It does not follow, however, that an active autolysin is necessarily produced whenever autolysis occurred. Thus lysates of *Escherichia coli* and *Salmonella* spp., obtained through the action of pyrophosphate or glycine or both, have no appreciable lytic activity. These two agents appear to act as inducers of autolysis and exert a synergistic effect, a fact which suggests that induction of autolysis may be realized in more than one way. Electrophoretic and serological studies also show that the products of lysis are different when different inducers are used (Salmon, 1957).

This picture of autolysis, natural or induced, was widely accepted, since it gave a coherent and reasonable explanation (although in rather vague terms) of many cases of autolysis, of the bacteriolytic activities shown by several antiseptics and antibiotics, even of the lysis of phage-infected bacteria. A deeper analysis of some particular cases has shown however that, in some instances, bacteriolysis is not primarily an enzymic phenomenon and, in some others, that it need not involve the hydrolytic enzymes or autolytic system of the cell. Thus Newton's work on the mode of action of polymyxin has clearly and elegantly demonstrated that fixation of the antibacterial agent upon the cytoplasmic membrane alters its permeability properties and promotes the leakage of cell material. No doubt, other agents, formerly believed to be inducers of autolysis, will be found to act in this much more direct way.

Meadow & Work (1956), Meadow, Hoare & Work (1957) and Rhuland (1957) have reported that *Escherichia coli* strain 173–25, a mutant requiring α,ϵ-diaminopimelic acid (DAP) for growth, lyses when cultivated in the presence of limiting amounts of this substance. Lack of enough DAP seems to prevent the organism from building its cell wall, and therefore the fragile naked cells which are formed undergo a primarily osmotic lysis. This mechanism, in which again the autolytic system plays at best a secondary role, will very likely be found responsible for other cases of autolysis.

In fact, a similar explanation was recently offered to account for the bacteriolytic activity of penicillin upon growing cells, a phenomenon which was formerly regarded by many as the result of an activation of the autolytic system. Following Lederberg's observation (1956, 1957) that *Escherichia coli* rods submitted to the action of the antibiotic in a hypertonic sucrose medium are transformed into large osmotically fragile bodies resembling protoplasts, it is now believed that penicillin interferes with cell-wall synthesis. The characterization of cell-wall components in the cultures of penicillin-treated bacteria (Park, 1952; Park & Strominger, 1957) is in good agreement with this interpretation. The precise point of action of the antibiotic has yet to be determined. It might prevent entry into the cell of a key substance necessary for cell-wall synthesis, or it might inhibit an enzyme taking part in this process. It might also disorganize the cytoplasmic membrane, a structure which is almost certainly involved in the formation of the cell wall. In fact, the permeability alterations which quickly follow upon the addition of penicillin to a bacterial suspension are rather in favour of this opinion. It is also possible that penicillin induces the production by the cell of a wall-destroying enzyme (Prestidge & Pardee, 1957). Again, this suggests that other agents which induce bacteriolysis may act according to a similar mechanism.

The role of the autolytic system in the lysis of phage-infected bacteria can be easily accepted in the case of those organisms which are known to autolyse readily. It is less acceptable for those many species which can be phage-infected but which are generally resistant to autolysis. Thus, the more or less specific lytic activities recorded in phage lysates of staphylococci by Gratia, by Wollman, and, more recently, by Wahl & Josse-Goichot (*lyse par entraînement*) or by Welsch, Cavallo & Cantelmo (lysis in the cold and collateral lysis of ultraviolet induced lysogenic staphylococci), were ascribed mainly to the normal autolytic system. In other cases, however, it appears that abnormal lytic systems are synthesized as a result of the viral infection. They may be agents distinct from the phage or components of the virus particle.

In the first group should be mentioned the phage-lysin of Sertic & Bulgakov, the virolysin of Ralston and collaborators, the prolysins of Huppert & Panijel, the endolysins to be discussed in this symposium by Jacob, and the streptolytic agent recently described by Maxted (1957). Their mode of action is generally unknown. It is permissible to mention with them the capsule-destroying enzyme synthesized by a strain of *Klebsiella pneumoniae* upon its being infected by a certain phage (Adams & Park, 1956; Park, 1956). It must also be recalled that procedures effective to induce lysogenic bacteria can promote the synthesis of a bacteriolytic agent (megacin) by some strains of *Bacillus megaterium* (Ivánovics & Alföldi, 1957). In the second group, one finds the endogenous prolysins of Panijel & Huppert (1957) and the wall-destroying enzyme of coliphages to be discussed later on by Weidel. From this brief review, it appears that all cases of autolysis, spontaneous or induced, cannot be explained by the activation of an autolytic system. This concept, however, should not be discarded outright since it probably still has a useful role to play.

Studying the controlled autolysis of pneumococci, Dubos observed that the first step of this process was a transformation of the organisms from the Gram-positive to the Gram-negative state. The change was enzymic in nature and the 'Gram-negativating' enzyme was supposed to be the one really initiating autolysis. This idea was taken up and elaborated upon by Stacey and his school. In fact, they devised a theory, supposed to be of general application, to account for the autolysis of Gram-positive organisms. According to that theory, which was later on expanded to explain the bacteriolytic activities of a *Streptomyces* sp., sensitive bacteria are first changed to Gram-negative cytoskeletons through the action of a nuclease, disintegration of the cells being thereafter brought about by two or more proteases. As a result of our own observations, we have never been convinced that loss of the Gram-positive character was necessary to make the cell sensitive to the properly lytic enzymes. Neither did the loss of Gram-positiveness appear to us as the result of the destruction of a substrate specifically linked with the Gram reaction. Rather we thought it merely expressed a general loss of ribonucleic acid with concomitant decrease of basophily. It is relevant to point out here that almost all methods used to obtain Gram-negative cyto-skeletons can be used just as well —in Gram-negative as well as in Gram-positive organisms—to remove cytoplasmic nucleic acids and thus show the bacterial nuclei.

Innumerable publications have reported the ability of various microorganisms to clarify turbid bacterial suspensions. Representatives of the genera *Bacillus* and *Streptomyces* are prominent in this respect. In many of these cases, very likely, an antibiotic is produced which, acting as inducer of autolysis or promoting permeability troubles, is responsible for the lytic process. In many others, one may be dealing with truly lytic agents, but in a few instances only has the mechanism of true heterolysis been studied and elucidated.

The production of lysozyme by *Micrococcus lysodeikticus* (Fleming & Allison), by *Streptomyces violaceus* (Kriss) and by *S. albus* (McCarty), has been reported, but without entirely satisfactory evidence. In contrast, the cell-wall dissolving enzyme obtained from a *Bacillus subtilis* by Richmond (1957) appears to be a true bacterial lysozyme.

A lytic agent, obtained from anaerobic autolysates of a *Bacillus subtilis* was obtained as a concentrated, but still far from pure, preparation by Nomura & Hosoda (1956*a*, *b*). According to its spectrum of activity, it is unlikely to be identical with lysozyme. It acts upon the cell walls of *B. subtilis* and *B. megaterium*, but has no action on *B. cereus*, *Micrococcus lysodeikticus*, *Sarcina lutea*, *Staphylococcus aureus* or *Escherichia coli*. However, it liberates reducing groups from its substrates and is considered as a polysaccharidase.

A cell-wall dissolving enzyme was recognized by Strange & Dark (1957) in the aqueous extracts of spores from *Bacillus cereus* and avirulent strains of *B. anthracis*. It acts upon the vegetative cell walls and spore coats of several bacilli. Unlike lysozyme, it is more active upon *B. cereus* than upon *B. megaterium* cell walls. It liberates from its substrates non-dialysable fragments containing all the 'basal' components of the bacterial cell wall. It should not be hastily concluded that the bacteriolytic activities of all *Bacillus* spp. are due to specific cell-wall digestion. In fact, Norris (1957) described the lysis of several *Bacillus* spp., mainly in the presence of thiomersalate, by a culture-filtrate from a *B. cereus*. Under its action, the sensitive organisms first lost their Gram-positive character, and then their capacity to retain the counterstain; but the cell wall could still be demonstrated by appropriate staining procedures.

The bacteriolytic properties of streptomycetes have been known since the early observations of Lieske and of Gratia & Dath. Their active filtrates (actinomycetin) are able to dissolve the suspensions of killed Gram-negative organisms and of some intact Gram-positive bacteria. The protein nature of the active principle(s) and the enzymic nature of the lytic process were demonstrated. It was believed, originally, that the agent acting upon heat-killed *Escherichia coli* (actinozyme) was involved, together with some additional principle, in the lysis of intact Gram-positive bacteria. This idea was also developed by Webb and his co-workers and more recently by Klinge (1955). However, Ghuysen has clearly shown that actinozyme and the staphylolytic system are distinct. They appear in, and disappear from, the culture medium at different moments. The optimal conditions for their production and for their activity, respectively, are different. They are adsorbed on, and eluted from, various substrates according to different physico-chemical conditions. From the culture-filtrates of a *Streptomyces albus*, McCarty isolated a streptolytic enzyme which was shown, by him and by Salton, to dissolve the cell wall of the sensitive organisms and was identified as a polysaccharidase distinct from lysozyme. Following this observation, our actinomycetin was found to contain, in addition to actinozyme, the staphylolytic system and several proteases, a streptolytic agent, possibly identical with McCarty's, and at least two pneumolytic principles. The staphylolytic system was isolated by Ghuysen and found to comprise two components: F1 and F2. The F1 component was obtained as a highly purified protein. Ghuysen and Salton studied the bacteriolytic spectra of these two agents. They are somewhat different, at least quantitatively. F1 and F2 act synergistically and their bacteriolytic activity is completely parallel to their digesting action upon the corresponding cell walls. They are different from lysozyme and from McCarty's enzyme, since they liberate from their substrates no reducing groups, no sugars, no amino sugars, but only unidentified small peptides, alanine and/or glycine, together with a large amount of undialysable material. They are regarded as peptidases and, to recall their origin as well as their bacteriolytic properties, they have been named actinolysopeptidases.

In conclusion, it can be stated that the few truly bacteriolytic agents as yet obtained in a purified state, act in destroying the cell wall of the sensitive cell, by degradation of either its polysaccharidic or its protein moiety.

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Lysis Resulting from Metabolic Disturbance

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When a suitable environment is maintained, there is every reason to expect that a bacterial culture can continue to grow indefinitely. Each part of each cell will increase in a balanced manner until division results in reproduction of the cellular unit. There is no evidence that a resting phase is necessary nor that a bacterial cell *must* sooner or later die. If a cell divides, then it cannot be said to die unless both of the progeny succumb since there is no indication of a mother-and-daughter relationship in bacterial reproduction. That individual cells do die may at present be interpreted in terms of chance or rather mischance—a local adverse condition (as in the 'spontaneous' induction of a lysogenic organism). In an unsuitable medium, growth may continue for a time or it may stop more or less abruptly; further, in each case the cells of the culture may remain in a state of suspended animation or they may rapidly lyse. A medium may be unsuitable by virtue of chemical or physical factors; or the presence of enzymes, viruses or bacteriocines; or factors which cause metabolic disturbance such as the absence of adequate nutrients or the presence of inimical drugs. This paper is concerned with lysis caused by metabolic disturbance.

The bacterial cell consists of a protoplast surrounded by a strong and rigid cell wall which maintains the form of the cell. The protoplast includes the cytoplasm and the nuclear apparatus (chromatinic body) and is bounded by a fragile protoplast membrane (cytoplasmic membrane, plasma membrane) which is the osmotic barrier of the cell. Lysis is said to occur when the structural integrity of the cell is destroyed. It can be observed macroscopically when a turbid suspension clears or microscopically when individual cells are seen to disintegrate, lose their density or change their staining characteristics. Thus it involves some change in the cell wall and/or protoplast membrane since these are the structures which maintain the form of the cell and the integrity of its internal constituents. Often the progress of lysis, like that of growth, is followed turbidimetrically. Alternatively, the leakage of intercellular substances into the surrounding medium can be studied. Changes which occur on the outside of individual bacterial cells can be investigated quantitatively by the method of micro-electrophoresis.

In order to define the nature of lysis it is necessary to know something of the composition and structure of the components involved, the cell wall and the protoplast membrane (see Fig. 1). Bacterial cell walls may comprise about 25 % of the dry weight of the cell and appear to be composed of mucopolysaccharide in Gram-positive organisms and this, together with lipoprotein in Gram-negative bacteria (see review by Work, 1957). The mucopolysaccharide usually contains *N*-acetylated hexosamine and muramic acid (3-*O*-α-carboxyethyl-hexosamine), one or more of the sugars, glucose, galactose, mannose, arabinose and rhamnose, and the amino acids, glutamic acid, and alanine together with lysine or diaminopimelic acid (DAP) and sometimes glycine or aspartic acid and traces of others. Where the point has been investigated it has been found that much of the glutamic acid and some of the alanine is of the D-configuration, the lysine L, and the DAP, meso- or LL. In Gram-negative bacteria the walls contain, in addition, up to 20 % lipid and also protein of conventional amino acid composition.

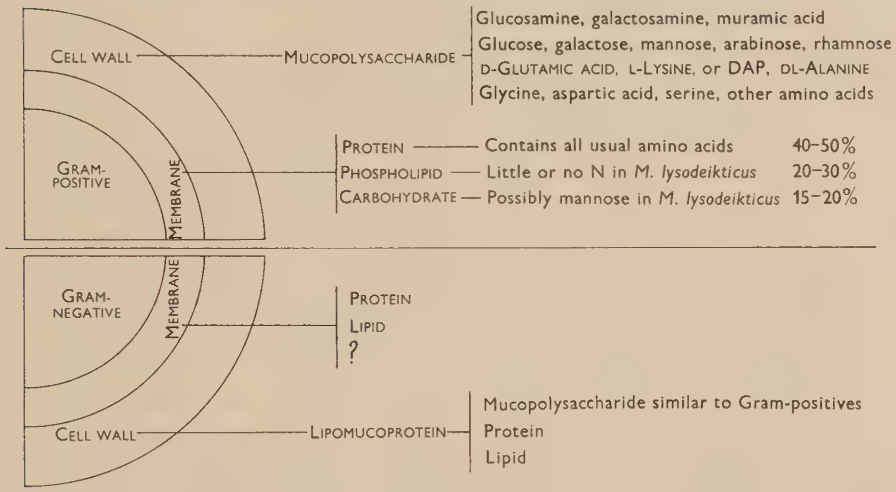
CELL WALL
MUCOPOLYSACCHARIDE
Glucosamine, galactosamine, muramic acid
Glucose, galactose, mannose, arabinose, rhamnose
D-GLUTAMIC ACID, L-LYSINE, or DAP, DL-ALANINE
Glycine, aspartic acid, serine, other amino acids
GRAM-POSITIVE
MEMBRANE
PROTEIN — Contains all usual amino acids 40–50%
PHOSPHOLIPID — Little or no N in M. lysodeikticus 20–30%
CARBOHYDRATE — Possibly mannose in M. lysodeikticus 15–20%
GRAM-NEGATIVE
MEMBRANE
PROTEIN
LIPID
?
CELL WALL
LIPOMUCOPROTEIN
Mucopolysaccharide similar to Gram-positives
Protein
Lipid

Fig. 1. Probable constitution of cell walls and protoplast membranes of Gram-positive and Gram-negative bacteria. (Data from several sources, see text.)

The protoplast membrane accounts for about 10 % of the dry weight of the cell and is usually considered to be lipoprotein, though few analyses have been made. That of *Staphylococcus aureus* is reported to be about 40 % protein and 22·5 % lipid (Mitchell & Moyle, 1956). The membrane of *Bacillus megaterium* is dissolved by lipase but not by ribonuclease, although it is claimed to contain 11 % ribonucleic acid (Vennes & Gerhardt, 1956). However, this figure is based solely on an orcinol reaction estimation which indicates only the presence of pentose. The protoplast membrane of *Micrococcus lysodeikticus* was found by Gilby & Few (personal communication) to contain 28 % lipid—mainly phospholipid of very low nitrogen content but also some carotenoid. The same membranes have been investigated by the writer who found 50 % protein (containing most of the usual amino acids) and about 16 % reducing sugar probably mainly mannose. Glucose, the only hexose present in the cell wall of *M. lysodeikticus* was absent and the hexosamine content was about 2·5 % as compared with 16 % for cell wall (McQuillen, unpublished observations). This reveals an unsuspected carbohydrate component of the protoplast membrane.

We will now consider specific instances of metabolic disturbance which can be rationalized in terms of what is known of the structures involved.

Unbalanced growth resulting from nutritional deficiency

Thymine deficiency in Escherichia coli. Strain 15_{T-} of *E. coli* requires thymine for normal growth, and omission of this substance from an otherwise adequate medium causes the organism to lose the power to form colonies at the rate of *c.* 90 %/generation time. This 'thymineless death' is accompanied by increase in turbidity of the culture, increase in length and diameter of the cells, and a doubling of the protein and ribonucleic acid (Cohen & Barner, 1954, 1956). The metabolic disturbance results in continued cytoplasmic growth without equivalent nuclear increase (for which thymine is required) and although this leads to rapid death it does not engender lysis.

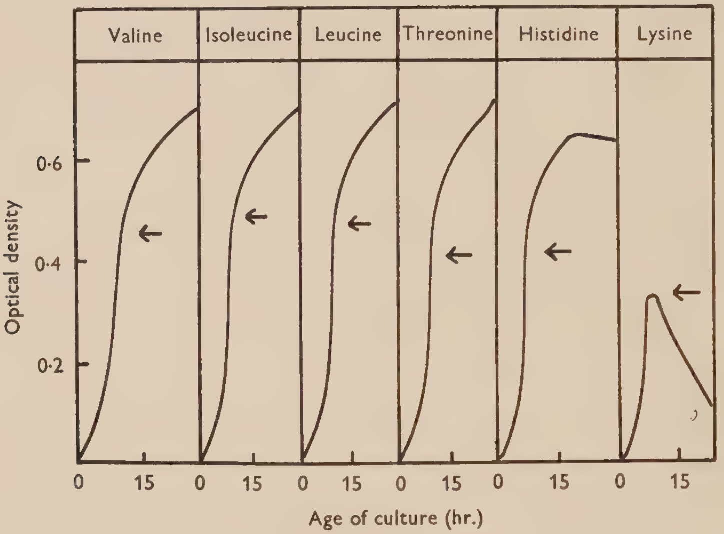

Fig. 2. Growth curves of *Streptococcus faecalis* 9790 in media limited by various amino acids. Arrows indicate where limiting amino acid is exhausted. (Data of Toennies & Shockman, 1953.)

Valine deficiency in Streptococcus faecalis. Strain 9790 of *S. faecalis* is exacting towards the amino acids, threonine, leucine, isoleucine, valine, methionine, arginine, histidine and lysine. Toennies and colleagues have studied the effects of limiting growth by each of these independently (Toennies & Gallant, 1949*a*, *b*; Toennies & Shockman, 1953; Shockman, Kolb & Toennies, 1957*a*, *b*). Only in the case of lysine limitation did lysis occur immediately after all the amino acid had been assimilated (see below). In all other cases there was a substantial increase in turbidity of the culture even after the limiting amino acid had been exhausted (Fig. 2). The effects of valine have been studied in more detail (Shockman *et al.* 1957*a*). After the medium was depleted of valine, growth of the culture ceased to be exponential but the optical density increased by a further 50 %. The dry weight of the cells went up by about 30 %, although the total N was little changed. However, the cell-wall fraction which in the exponential phase accounted for 13 % of the total N, had increased to represent 24 %. Moreover, of the cellular amino acids only lysine had altered appreciably (increase of 27 %) and lysine, together with glutamic acid and alanine, is a constituent of the cell wall, whereas valine and the other amino acids are not. Rhamnose and glucosamine, two compounds present almost exclusively in the wall, were also found to have nearly doubled in amount during the post-exponential phase. It would seem that limitation of valine (and possibly several other amino acids) results in unbalanced growth such that while cellular protein cannot be synthesized, bacterial cell wall can continue to be formed and may almost double in amount. As with limitation of nuclear development this does not, however, lead to lysis.

Lysine deficiency in Streptococcus faecalis. When with the same strain 9790 of *S. faecalis*, lysine was the limiting amino acid, growth was exponential until all the amino acid had been used and then rapid lysis occurred (Toennies & Gallant, 1949*b*). The requirements for onset of lysis were reported to be: (*a*) depletion of lysine from the medium; (*b*) a pH value near neutrality; (*c*) high concentration of phosphate (0·3 M). Recently, the matter has been re-investigated with some interesting developments (Shockman *et al.* 1957*b*): (*a*) cells need not be taken from lysine-depleted media; when taken from the exponential phase they are 'lysis-prone' whether lysine or valine would ultimately limit growth. (*b*) Cells grown at lower pH values can be induced to lyse when the pH is raised. (*c*) High phosphate concentration is not essential for lysis: it can occur in lysine-free phosphate buffer growth medium or more rapidly in 0·3 M-phosphate buffer or in M-NaCl. However, lysis does not occur in distilled water.

Briefly the situation can be summarized as follows: *Streptococcus faecalis*, strain 9790, when removed from the exponential phase of growth or at the end of growth limited by lysine deficiency (but not other amino acids) shows a marked tendency to lyse in salt or buffer solutions of pH value near neutrality. Should growth be limited by an amino acid such as valine which, unlike lysine, is not a component of the cell wall, then after the exponential phase there follows a period during which cell-wall material increases without increase in cytoplasmic protein.

Diaminopimelic acid deficiency in Escherichia coli. Lysis caused by amino acid limitation has also been studied by Meadow & Work (1956), Meadow, Hoare & Work (1957) and by Rhuland (1957). Meadow used three mutant strains of *E. coli* ACTC 9637: 26-26 which requires lysine, 173-25 which has an absolute requirement for diaminopimelic acid (DAP) and is stimulated by further addition of lysine, and D (derived from 173–25) which requires DAP but is not stimulated by lysine. It is probable that in *E. coli* lysine is normally formed by decarboxylation of DAP:

$$\mathrm{COOH.CHNH_2CH_2CH_2CH_2CHNH_2COOH} \rightarrow \mathrm{CH_2NH_2CH_2CH_2CH_2CHNH_2COOH + CO_2}.$$

The lysine-requiring mutant 26-26 grew well in minimal media + lysine and showed no tendency to lyse. Mutant D behaved in the same way when DAP was added to the basal medium. However, when mutant 173-25 was grown in media containing excess lysine but limiting DAP, lysis of the culture began immediately after attainment of maximum optical density. Moreover, a similar sequence of events followed when mutant D was grown in adequate medium+lysis. The lysine of mutant 173-25 was prevented by addition of lanthionine to media containing lysine and DAP or by replacing the lysine by α-*N*-acetyl lysine. Rhuland (1957) showed that lysis of mutant 173-25 in limiting DAP was a function of the lysine concentration and that it could be overcome by adding more DAP.

As a result of the findings of Meadow & Work (1956), it was apparent that it might be possible to obtain *Escherichia coli* deficient or wholly lacking in cell wall (i.e. protoplasts) by DAP limitation. It was already known that in *Bacillus megaterium* DAP is present as a structural component only in the cell wall and that removal of the wall by controlled digestion with lysozyme yields protoplasts still having most of the attributes of living organisms (McQuillen, 1956). Dr Meadow kindly agreed to collaborate with the writer in an attempt to see whether or not lysis of the mutant 173–25 in media deficient in DAP was due to formation of protoplasts followed by lysis of these fragile structures. Using continuous phase-contrast microscopy of micro-colonies in droplets of media suspended from a cover glass and immersed in liquid paraffin, we were able to watch DAP-limited *E. coli* develop into spherical forms which subsequently lysed to leave 'ghosts' of the same shape (McQuillen & Meadow, 1956, unpublished observations mentioned in Meadow *et al.* 1957). It is possible but not certain that these spherical forms which are osmotically sensitive are devoid of cell wall.

More recently, the writer has been able to obtain stable preparations of spherical and near-spherical form by growing heavy suspensions of *Escherichia coli* 173-25 in media lacking DAP but containing 20 % sucrose to give osmotic support (see Pl. 1). This technique is analogous to that used by Lederberg (1956) for obtaining 'protoplasts' of *E. coli* by penicillin treatment (see below). Bauman & Davis (1957) have independently shown that DAP-deprivation gives rise to 'protoplasts' of the same mutant of *E. coli*.

Unbalanced growth resulting from drug action

Antibiotics, including chloramphenicol. It is now well established that chloramphenicol inhibits the synthesis of protein in many species of bacteria. The inhibition is more or less immediate and can result in unbalanced increase of components such as nucleic acids and capsular material (Gale & Folkes, 1953; Hahn, Wisseman & Hopps, 1954; Wisseman, Smadel, Hahn & Hopps, 1954) without concomitant formation of protein. It has recently been suggested that in *Escherichia coli* the nucleic acid formed in the presence of chloramphenicol is abnormally unstable and rapidly breaks down on removal of the antibiotic (Neidhardt & Gros, 1957). However, physiologically active deoxyribonucleic acid can be formed in phage-infected bacteria in the presence of chloramphenicol (Hershey & Melechen, 1957) and the ribonucleic acid formed in *Staphylococcus aureus* treated with chloramphenicol seems to play a role in the subsequent formation of protein when the drug is removed (Gale & Folkes, 1953). Whatever its mode of action, it is true to say that in general chloramphenicol does not cause rapid lysis of bacteria.

That there is no immediate change in the surface structures is also shown by micro-electrophoresis studies. The effects of a range of antibiotics on *Staphylococcus aureus* were determined in washed suspensions and in growing cultures (McQuillen, 1950, unpublished observations). At 100 μg./ml. the only antibiotic which had an *in vitro* effect was streptomycin (Table 1), which was shown to adsorb to the surface of bacteria (McQuillen, 1951*b*). Growth of *S. aureus* for up to 60 min. in chloramphenicol, chlortetracycline or oxytetracycline at 10 μg./ml. did not result in any detectable change in cell surface (Table 2). This can be contrasted with the action of penicillin (see below) which was apparent within a few minutes (McQuillen, 1951*a*).

Table 1. *Effect of antibiotics on washed suspensions of* Staphylococcus aureus

Electrophoretic mobilities determined in phosphate buffer (0·003 M, pH 7·3), containing glucose (1 mg./ml.) and, where indicated, antibiotic (100 μg./ml.). (Data of McQuillen, 1950, unpublished.)

Antibiotic	Electrophoretic mobility (μ./sec./volt/cm.)
None	2·68
Oxytetracycline	2·69
Chlortetracycline	2·68
Chloramphenicol	2·66
Penicillin	2·62
Bacitracin	2·74
Streptomycin	2·07

Table 2. *Effect of antibiotics on growing cultures of* Staphylococcus aureus

Electrophoretic mobilities determined in phosphate buffer (0·003 M, pH 7·3) containing broth and, where indicated, antibiotic. (Data of McQuillen, 1950, unpublished.)

Antibiotic	Concentration (μg./ml.)	Electrophoretic mobility in μ./sec./volt/cm. after growth for 5 min.	30 min.	60 min.
None	—	2·07	2·10	2·05
Oxytetracycline	10	2·11	2·11	2·10
Chlortetracycline	10	2·06	2·08	2·05
Chloramphenicol	10	2·05	2·08	2·04
Penicillin	1	1·90	1·69	1·55

Penicillin. Although it has long been known that penicillin causes lysis of susceptible bacteria, there have been many theories about its mode of action; recently additional evidence has been obtained for one of these. Gardner (1940) discussed the morphological effects of penicillin and pointed out that *Escherichia coli* and other Gram-negative rods 'often showed grotesque giant forms due to autolytic swelling'. In staphylococci the 'change takes the form of spherical enlargement and imperfect fission'. Duguid (1946) wrote 'the morphological changes produced by the lower penicillin concentrations...the ready occurrence of swelling and protoplasmic protrusion, suggest that penicillin...interferes specifically with formation of the outer supporting cell wall, while otherwise allowing growth to proceed until the organism finally bursts its defective envelope and undergoes lysis'.

Many studies have shown that penicillin is bactericidal only when the organisms are in an environment in which growth can occur. McQuillen (1951*a*) found that high concentrations of penicillin had no effect on the surface density of charge of *Staphylococcus aureus* and *Escherichia coli* suspended in buffer. In growth medium, or in buffer supplemented with a small amount of broth (1/100 dilution) addition of 0·1 unit penicillin/ml. initiated a change in the surface characteristics of *S. aureus* which could be detected within a few minutes; higher concentrations of penicillin provoked a similar change in *E. coli*. It was concluded that changes in the surface of susceptible bacteria were the earliest effects of penicillin then reported.

Although, by and large, Gram-positive bacteria are more sensitive to penicillin than are Gram-negative bacteria, the latter are by no means all resistant. *Escherichia coli* B, for instance, has an inhibition coefficient of 5 μg./ml. when growing in a glucose + NH_3 + salts medium. Harrington (1954) surveyed the effects of penicillin on this organism. He found that after adding 5 μg./ml. or more of the drug to a growing culture, the turbidity increased for 15–30 min. and then lysis ensued (Fig. 3). Neither protein nor nucleic acid synthesis was much affected during this pre-lytic period but the onset of lysis coincided with cessation of both, and with release into the medium of protein and 260 mμ.-absorbing material which was not precipitable by trichloroacetic acid. The course of lysis could be followed by measuring the fluorescence on addition of *N*-tolyl-α-naphthylamine-8-sulphonic acid, which combined with the released protein to form a fluorescent conjugate.

It was found that Mg^{++} had a dramatic effect in preventing lysis. Addition of 0·02 M-Mg^{++} to a culture inhibited by 5 μg. penicillin/ml. allowed normal increase of turbidity and prevented release of 260 mμ.-absorbing material for a 2 hr. period (Fig. 4). This protection was nullified by further addition of citrate and in the absence of additional Mg^{++}, citrate enhanced the lytic effects caused by penicillin. Conversely, addition of Mg^{++} to cells in the presence of citrate and penicillin afforded protection against lysis (Fig. 5). It is interesting that later Weibull (1956) reported that 0·01 M-Mg^{++} preserved the integrity of protoplast membranes of *Bacillus megaterium* and that Lederberg (1956) found a high concentration of Mg^{++} necessary for formation of stable 'protoplasts' of *Escherichia coli* by growth in broth containing penicillin (see below).

A striking demonstration of the necessity for growth before penicillin can exert its effect was also given by Harrington (1954). He showed that addition of an amount of chloramphenicol which just inhibited protein synthesis also prevented lysis caused by penicillin. Moreover, when lysis had begun it could be arrested by addition of chloramphenicol (Fig. 6). In these experiments it was also found that penicillin had no effect on nucleic acid synthesis in cells prevented from making protein by presence of chloramphenicol.

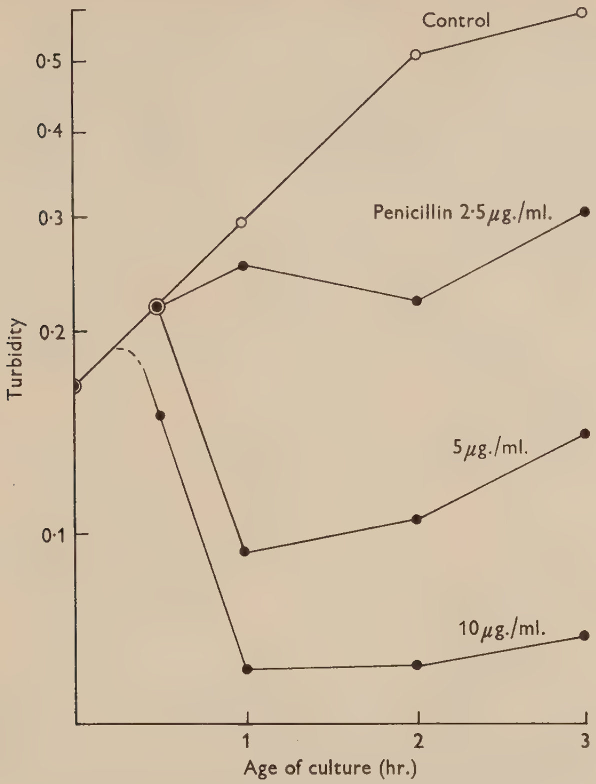

Fig. 3. Growth curves of *Escherichia coli* B in the presence of 0, 2·5, 5 and 10 μg./ml. penicillin. (Data of Harrington, 1954.)

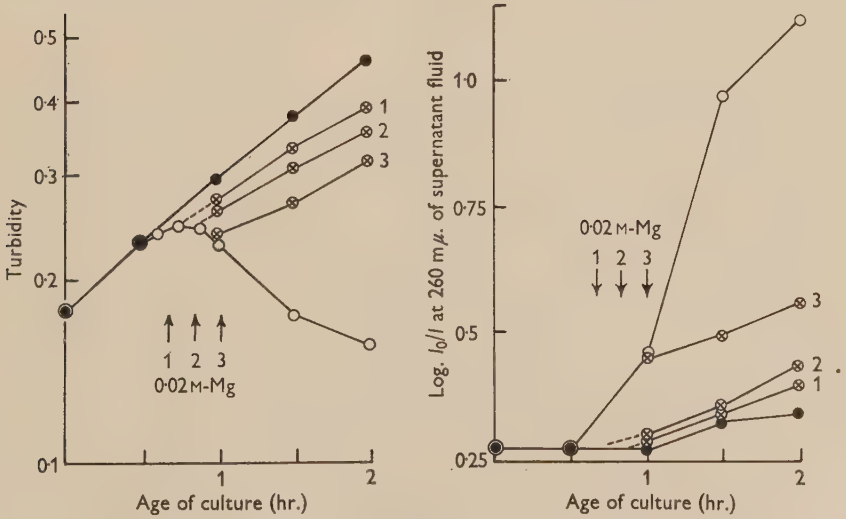

Fig. 4. Growth curves of *Escherichia coli* B and release of 260 mμ.-absorbing material into supernatants of cultures. ●—●, Control; ○—○, 5 μg./ml. penicillin at time 0; ⊗—⊗, 5 μg./ml. penicillin at time 0 and 0·02 M-Mg^{++} added at time 40, 50 or 60 min. (Data of Harrington, 1954.)

Three groups of workers (Lederberg, 1956; Liebermeister & Kellenberger, 1956; Hahn & Ciak, 1957) have now independently shown that under appropriate conditions, growth of *Escherichia coli* in the presence of penicillin can result in the formation of osmotically-fragile spherical bodies which resemble the protoplasts which can be obtained from certain Gram-positive bacteria by

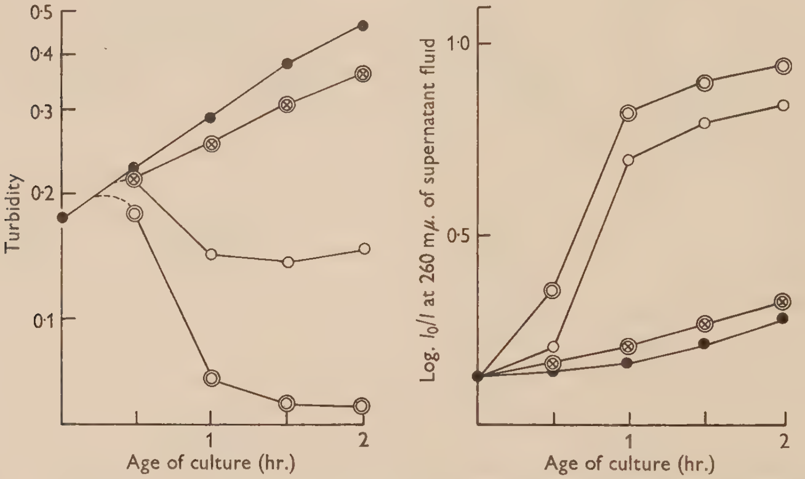

Fig. 5. Growth curves of *Escherichia coli* B and release of 260 mμ.-absorbing material into supernatant fluid of cultures. ●—●, Control; ○—○, 5 μg. penicillin/ml. at time 0; ◎—◎, 5 μg. penicillin/ml. and 0·04 M-citrate at time 0; ⊗—⊗, 5 μg. penicillin/ml. and 0·04 M-citrate at time 0, followed by 0·03 M-Mg⁺⁺ at time 10 min. (Data of Harrington, 1954.)

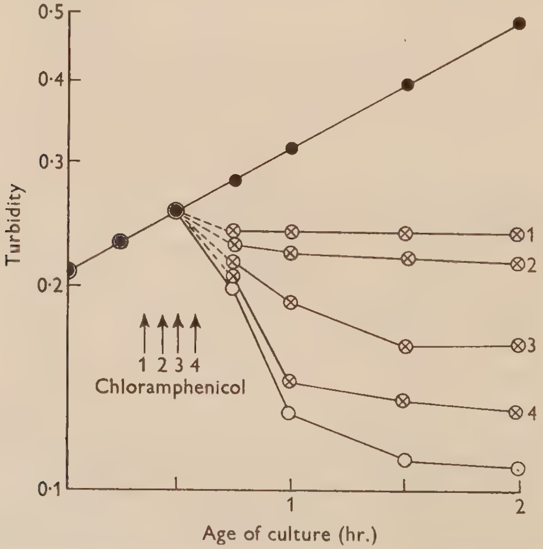

Fig. 6. Growth curves of *Escherichia coli* B. ●—●, Control; ○—○, 5 μg. penicillin/ml. at time 0; ⊗—⊗, 5 μg. penicillin/ml. at time 0 and 10 μg. chloramphenicol/ml. added at time 20, 25, 30 or 35 min. (Data from Harrington, 1954.)

digestion of the cell wall with lysozyme (Weibull, 1956; McQuillen, 1956). A fully-grown broth culture of *E. coli* is diluted about fourfold with fresh broth containing 100–1000 μg. penicillin/ml., 20 % sucrose and 0·2 % magnesium sulphate. After aerated growth for *c.* 2 hr., the rods become converted into spherical 'protoplasts'. Dilution of the sucrose resulted in immediate lysis.

The writer has confirmed these findings and has also determined that the 'protoplasts' contain very much less DAP in their trichloroacetic acid-precipitable fraction than do intact cells of *Escherichia coli*. They resemble quite remarkably the 'protoplasts' of *E. coli* formed by deprivation of DAP (see Pl. 1). There is, however, still no certainty that the entire cell wall is absent from these Gram-negative 'protoplasts' and the unqualified term protoplast should not be used until this is established.

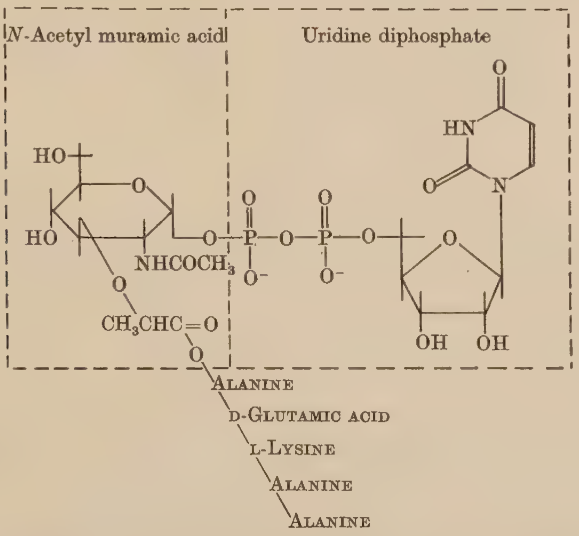

Fig. 7. Suggested constitution of uridine nucleotide which accumulates when *Staphylococcus aureus* grows in the presence of penicillin. (After Park & Strominger, 1957.)

A biochemical interpretation of many of the above findings has recently been assembled by Park & Strominger (1957). Park (1952) had found that treatment of *Staphylococcus aureus* with penicillin resulted in the accumulation of uridine nucleotides, including one linked to a peptide containing alanine, glutamic acid and lysine. It is now suggested that the structure of this nucleotide might be as shown in Fig. 7. The cell wall of *S. aureus* has also been analysed and shown to contain several of the same components and in the same relative proportions as in this nucleotide (Table 3). The substances common to both are *N*-acetyl-muramic acid (the 'unknown amino sugar' present in the cell walls of many bacterial species), D-glutamic acid, L-lysine and DL-alanine. Park & Strominger (1957) suggest that the uridine nucleotide is an intermediate in cell-wall synthesis and that penicillin prevents its utilization, and this 'interferes with the maintenance of the cell wall or its synthesis'.

Table 3. *Comparison of analyses of cell wall and a uridine nucleotide which accumulates in penicillin-treated* Staphylococcus aureus

The data are from Park & Strominger (1957) and are expressed as approximate relative molar proportions.

Component	Cell wall	Uridine nucleotide
Uracil	0	1
Ribose	0	1
N-Acetyl muramic acid	1	1
D-Glutamic acid	1	1
L-Lysine	1	1
DL-Alanine	3	3
Glucosamine	2	0
Glycine	*c.* 4	0

DISCUSSION

A number of examples of metabolic disturbance have been described and it is clear that only in some is lysis an early consequence. It is possible to restrict nuclear development while allowing protein and ribonucleic acid to increase and, equally, it is possible to prevent protein synthesis while allowing nucleic acid and cell-wall formation to continue; in neither case does lysis necessarily result. Other derangements, however, result either in 'protoplast' formation or in lysis, depending on whether or not osmotic protection is afforded by, for example, solutions of high sucrose concentration. In these cases it seems that lysis is a direct result of the failure of the cell wall to support the osmotic barrier (the protoplast membrane). It is likely that in all the cases described above, lysis results from an active process of unbalanced growth of cytoplasm.

In the case of the mutant of *Escherichia coli* which requires DAP and lysine (173–25) restriction of DAP in the presence of adequate lysine will prevent cell-wall synthesis while allowing continued cytoplasmic protein synthesis. On the other hand, mutant D which needs only DAP and can derive its lysine from DAP, will have a source of the cell-wall component (DAP) and of lysine for its protein so long as any DAP remains (Fig. 8). Thereafter, it can make neither cell wall nor cytoplasmic protein. The situation is altered, however, when exogenous lysine is supplied. In this case cytoplasmic protein can increase after cell-wall formation is limited by exhaustion of DAP. Thus lysis might be expected and was found. The inhibition of lysis of *E. coli* mutants 173–25 and D by addition of lanthionine to media containing lysine and limiting DAP, results in a growth response qualitatively and quantitatively very similar to that of mutant D in the absence of lysine and lanthionine. This might be explained if lanthionine were found to inhibit the utilization of exogenous lysine. It was inferred by Meadow *et al.* (1957) that lanthionine does not inhibit DAP decarboxylation* because cystine does not and because both substances inhibit the growth of DAP-requiring mutants competitively in the absence of lysine. This might indicate also an inhibition of utilization of endogenously formed lysine. The finding that the lysine-requiring mutant 26–26 of *E. coli* does not lyse when lysine is limited is not surprising if this thesis of unbalanced growth be valid. Lysine is a component of both the cytoplasmic protein and the cell wall protein of *E. coli* and its restriction would not, *a priori*, be expected to allow continued formation of one rather than the other (Fig. 8).

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* This has now been confirmed experimentally (Dr Meadow, personal communication).

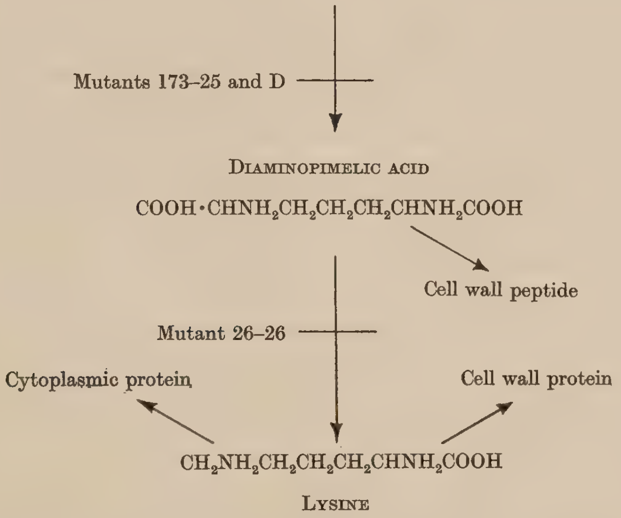

Fig. 8. Interrelationships and functions of diaminopimelic acid and lysine in *Escherichia coli*, ACTC 9637, and its mutants. Mutant 26–26 requires lysine, mutant D requires diaminopimelic acid, and mutant 173–25 has an absolute requirement for diaminopimelic acid and a partial requirement for lysine.

The situation with the Gram-positive *Streptococcus faecalis* is rather different. Here the cell wall is simpler in constitution; there is little lipid and no protein. The peptide component of the mucopolysaccharide contains only a few amino acids among which are D-glutamic acid, D- and L-alanine and L-lysine. Limitation of growth by an amino acid not present in the wall (e.g. valine) apparently allows excess wall synthesis after cytoplasmic protein synthesis ceases. (It is puzzling that Shockman *et al.* (1957*a*) did not find an increase in the glutamic acid and alanine as well as in lysine during the post-exponential phase.) When, on the other hand, lysine is restricted, lysis ensues. However, it was found that cells taken from the exponential phase of growth in valine-limited media were also prone to lyse. This is similar behaviour to that observed in *Staphylococcus aureus* by Mitchell & Moyle (1957). Indeed the isolated walls of this organism are reported to undergo autodigestion. It cannot be said from the evidence available whether or not in the case of *S. faecalis* an autolytic process is involved—possibly a running backwards of reactions normally concerned in cell-wall synthesis. This may be a somewhat general phenomenon in cells taken from the phase of most rapid growth. Certainly organisms are known to produce enzymes which degrade cell walls, e.g. *Bacillus subtilis* (Nomura & Hosoda, 1956; Richmond, 1957). On the other hand, it appears significant that only with lysine-limited *S. faecalis* does lysis follow immediately after maximum culture density is reached and that lysine is one of the few amino acids present in the cell wall.

The action of penicillin in causing lysis of Gram-positive and Gram-negative organisms depends on the occurrence of growth or, more specifically, of protein synthesis. Under such conditions there is a change in the nature of the surface of the cells within a few minutes of the addition of the drug. In the case of *Staphylococcus aureus* there accumulates a compound containing muramic acid and amino acids which is believed to be a precursor of part of the cell wall. Although the cell walls of Gram-negative organisms are more complex, that of *Escherichia coli* contains muramic acid, glucosamine, alanine, glutamic acid and diaminopimelic acid. These substances are specifically released from the cell wall by the action of bacteriophage (Weidel, this Discussion) suggesting that they occur as a structural unit.

Several Gram-negative bacteria can be converted, by growth in media of high osmotic pressure in the presence of penicillin, into protoplast-like forms in which the cell wall is either absent or weakened. In the case of *Escherichia coli* these appear almost identical to the 'protoplasts' formed by DAP-deprivation. Protoplasts of *Bacillus megaterium* entirely lacking cell wall are capable of protein and nucleic acid synthesis, enzyme formation, bacteriophage growth and even growth and division (McQuillen, 1956). They can, moreover, synthesize DAP, although they excrete it (or a derivative) rather than incorporate it into cell wall. The protoplast-like forms of *E. coli* have also been studied by the writer and have considerable synthetic abilities. They are able to increase in size and to synthesize enzymes. Lederberg (1956) reported that about half these protoplast-like forms are capable of reverting to rods in the absence of penicillin. It appears justified at present to conclude that the lysis caused by penicillin treatment results from an interference with cell-wall formation without concomitant cessation of certain other cellular activities. This unbalanced growth puts a stress on the protoplast membrane which it cannot withstand except in media of high osmotic pressure.

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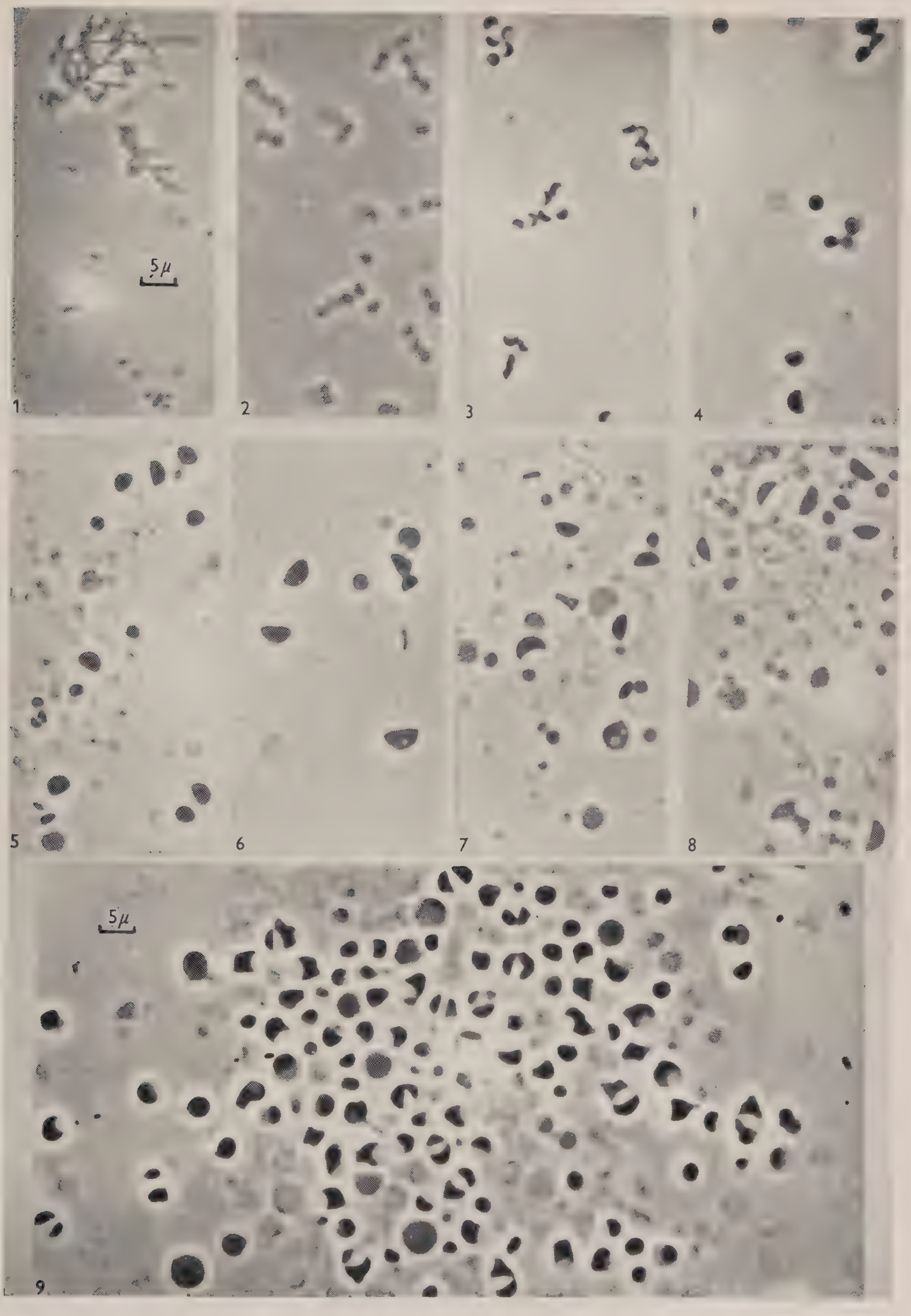
EXPLANATION OF PLATE

Figs. 1–8. Phase-contrast photomicrographs of *Escherichia coli* B growing in broth containing sucrose (15 %, w/v) and penicillin (100 μg./ml.). Fig. 1 at time 0, figs. 2–4 at times 60 to 90 min., fig. 5 at time 120 min., and figs. 6–8 at times 180 to 240 min. Magnification, × 1000.

Fig. 9. Phase-contrast photomicrograph of *Escherichia coli* 173–25 growing in broth containing sucrose (15 %, w/v) but no diaminopimelic acid (to which this mutant is exacting). The cells are similar to those in Fig. 1 at time 0. Fig. 9 shows the appearance at time 180 min. Magnification, × 1000.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K. McQuillen—Lysis resulting from metabolic disturbance. Plate 1

(*Facing p.* 512)

WEIDEL, W. & PRIMOSIGH, J. (1958). *J. gen. Microbiol.* **18**, 513–517

Biochemical Parallels between Lysis by Virulent Phage and Lysis by Penicillin

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In the course of our work on the biochemistry of receptor-active components of the *Escherichia coli* B cell wall, bacteriophages quite generally proved to be very useful and sensitive tools for helping to dissect the wall into many different, functionally intact, macro-components. For this discussion the most relevant result of these dissections is the finding that the coli wall is composed of two layers: one is lipoprotein and amounts to about 80 % of the dry weight of the cell wall; the other layer is characterized by a large content of saccharides + lipids + a few typical amino acids which later will be considered more closely. This 20 % layer we have called the lipopolysaccharide layer (Weidel, Koch & Lohss, 1954).

After removing the lipoprotein layer from isolated cell walls by 90 % phenol, in which it is soluble, the remaining lipopolysaccharide, when viewed under the phase-contrast microscope, appears largely in the form of extremely thin-walled transparent rods. Obviously the lipopolysaccharide layer is rod-shaped as such and forms the supporting framework of the coli wall to which it lends rigidity. The lipoprotein cannot have either of these two properties by itself because of its great plasticity which becomes directly noticeable after removing by dialysis the phenol in which it was dissolved.

The relative positions of these two layers can be ascertained by using phages as indicators. Before phenol treatment the coli wall adsorbs the coliphages T2, T4 and T6. After removing the lipoprotein layer with phenol, T2 and T6 are no more adsorbed by the remainder of the wall, i.e. the lipopolysaccharide, whereas the adsorbability of T4, as measured by rate of adsorption, is markedly increased. One is led to the conclusion that additional receptor sites for T4, which all lie in the polysaccharide layer, must have been uncovered and made accessible by removing the lipoprotein. Since the phage particles in these tests can interact with the wall from the outside only, it would follow that the outside is formed largely by the lipoprotein layer which contains the receptor sites for T2 and T6 and covers a great deal of the T4-sites actually present in the lipopolysaccharide layer.

With this picture in mind we now turn to the mechanism of destruction of the double-layered coli wall by certain virulent phages. As early as 1949 we were able to show on electron micrographs that coliphage T2, after adsorption to coli cell walls, causes structural changes within, and release of material from them (Weidel, 1951). This phenomenon seemed to indicate a specific enzymic activity of the phage particle which might well explain the more general phenomena of virus penetration and lysis 'from within' or 'from without'.

In order to specify the assumed enzymic activity of T2-particles, the technical problem of labelling coli walls in a really convenient way had first to be solved. The most obvious technique of using isotopes for labelling was employed by Kozloff and co-workers (Barrington & Kozloff, 1954) who were able to confirm the effect in question and to establish some quantitative relationships between number of phage particles adsorbed/cell wall and amount of wall material released. However, in the course of our investigations on virus receptors, we had come upon a much more tractable method of labelling. Coli cell walls react easily with dinitrofluorobenzene, thus taking on an intense yellow colour, yet without losing their ability specifically to adsorb coliphages T2, T4 and T6, which by the way indicates that for these phages primary amino groups in the wall are not essential to constitute active receptor sites. Upon adsorption, these phage types release soluble yellow material from the yellow di-nitrophenylated DNP walls, and quantitative measurements of enzymic action under different conditions are thus made very easy. All one needs is a photometer. With the help of such dyed-in-the-wool coli cell walls we were able to establish three facts, among others, which concern us here (Koch & Weidel, 1956):

(1) The phage enzyme is not type specific in contrast to the type-specific dependence of phage on their different receptors. Coliphages T2 and T4, for instance, attach to chemically very different sites of the coli wall, yet their enzymes remove the same material from the wall.

(2) The receptor sites are therefore obviously not the point of attack of the phage enzyme.

(3) The soluble wall material which appears after phage enzyme action contains three amino acids in comparatively large amounts, namely alanine, glutamic acid and diaminopimelic acid.*

The next step was to find which of the two layers of the coli wall contained especially high amounts of the three amino acids mentioned. No doubt it must be this layer which serves as 'substrate' for the phage enzyme.

Plate 1, fig. 1, shows a two-dimensional paper chromatogram of hydrolysed wall lipoprotein, the phenol-soluble 80 % layer. It offers a complete spectrum of amino acids typical of any average protein and, moreover, diaminopimelic acid is missing. Certainly this cannot be the layer which is attacked by phage enzyme.

Plate 1, fig. 2, shows the chromatogram of hydrolysed lipopolysaccharide, the 20 %-layer. Diaminopimelic acid, glutamic acid and alanine form especially large spots. In addition, there is a rather substantial spot of glucosamine, and spots of glycine and lysine are also well marked. Other amino acids are either missing or present in very much smaller amounts. Yet I wish to point out one of the weaker spots, no. 16; this is muramic acid as far as can be said from comparisons with original muramic acid kindly supplied to us by Dr Strange. Its presence will prove to be quite significant.

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* The latter compound we thought at first to be cystine, judging merely from the R_F-value we found for the DNP-derivative; we are very grateful to Dr Elizabeth Work for drawing our attention to the possibility of a mistake we had made here.

Undoubtedly the substrate for the phage enzyme has now been found. But the chromatogram of the lipopolysaccharide presents by itself a rather unexpected discovery. Probably any of the specialists concerned with cell walls would ascribe this chromatogram to a Gram-positive cell wall. Very remarkably, a typically Gram-negative organism like *Escherichia coli* B hides under a thick cover of lipoprotein a wall which is typically 'Gram-positive' in composition.

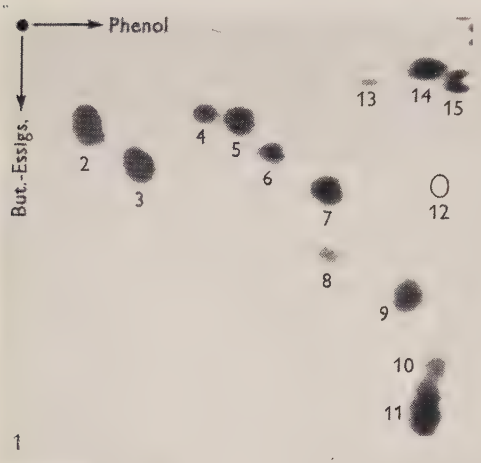
It remains to be seen now whether phage enzyme really does act directly upon the 'Gram-positive' lipopolysaccharide layer. Whole phage cannot be used very conveniently for this experiment, since it is impossible to remove the protein coat of the phages from the mixture after their enzyme has acted on the wall layer. Yet the coats have to be removed, or else their amino acids unavoidably obscure the chromatograms later on. A method for obtaining phage-enzyme free from enzymically-inactive phage protein had therefore to be devised. With the help of DNP walls it was found that enzyme is set free from phage particles by freezing and thawing them under certain conditions (Weidel & Primosigh, 1957). Enzyme solutions prepared by this simple procedure from coliphage T2 contain very little protein but sufficiently high enzymic activity. When mixed with lipopolysaccharide, the enzyme solution dissolves about one-third of the material (dry weight) quite rapidly at 37°, then stops acting further on it. The rest is left in insoluble form and can be removed from the mixture by centrifugation. Both residue and supernatant fluid are then hydrolysed and chromatographed.

Plate 1, fig. 3, shows a chromatogram of the hydrolysed soluble split-product. The complex or complexes cut out from the lipopolysaccharide layer by phage enzyme contain all the amino acids and amino sugars which have been seen to characterize this layer: diaminopimelic acid, glutamic acid, alanine, glucosamine, muramic acid, and small amounts of glycine and lysine. The insoluble residue is almost free from all these components except glucosamine, part of which remains with it. The chromatogram in question, when run at the proper relative concentration, is practically a blank after spraying with ninhydrin. This enzyme-resistant residue is made up mainly from lipid, glucose, L-gala-D-mannoheptose and some glucosamine, and it still carries the receptor sites for coliphage T4 in completely active form. Although the enzyme removes a very substantial amount of material from the lipopolysaccharide layer, it leaves its typical receptor sites untouched. The point of attack of the enzyme appears to be a glycosidic bond, since the soluble split-product has reducing power which is neither found with the insoluble residue nor with the lipopolysaccharide before enzyme treatment.

After having taken apart the coli cell wall in the manner described it may be attempted now to rebuild it from the pieces and to obtain some insight into their structural and functional relationships. As will have been noticed, the qualitative composition of the complex or complexes which phage enzyme cuts out from the coli wall's deeper layer, is most strikingly paralleled by the composition of one of the uridine-5′-pyrophosphate-linked complexes excreted by Park's penicillin-inhibited *Staphylococcus aureus* (Park, 1952). In addition, one has to take into account that both penicillin and the enzyme of certain virulent phages, under appropriate conditions, cause coli cells to lyse. A closer examination of these conditions is very revealing. Whereas lysis by penicillin requires the cells to be in a state of active metabolism, lysis by phage enzyme on the contrary occurs best when the cells are prevented from metabolic activity. Obviously then, lysis in both cases must be linked to one and the same key component of the cell wall, namely the complex containing the three characteristic amino acids and muramic acid. Under the influence of penicillin, instead of being built into the growing wall, this key component accumulates unused (Park & Strominger, 1957) and all newly formed pieces of cell wall must thus primarily have abnormal properties. Phage enzyme, on the other hand, removes the key component from normal walls, and if the damage cannot be repaired by replacing the loss, the wall at the point of attack must secondarily acquire the same kind of abnormality which, as can be seen in the phase-contrast microscope, consists in a loss of rigidity. Spherical protrusions appear on the rod-shaped cells which eventually are transformed into large bubbles and may burst in a medium of low osmotic pressure.

Therefore, as the lipopolysaccharide layer of the coli wall was said to be responsible for the rigidity of the whole wall, the often mentioned complexes in turn must be responsible for the rigidity of the lipopolysaccharide layer of which they are an integral part. One may assume that they form bridges between islets of co-polymerized glucose, heptose and lipid, to which they are attached by glycosidic bonds. Schematically, the lipopolysaccharide layer could thus be visualized in the manner shown in Pl. 1, fig. 4. Since phage enzyme not only breaks the bridges but appears to remove them completely, one might expect them to carry the same functional group at both ends. Otherwise one would have to attribute two different specificities to the enzyme, which seems improbable. Sequence analysis of the complexes will clear up this point.

To complete the picture of the coli wall, one only has to smear now a thick layer of plastic lipoprotein over the structure drawn in Pl. 1, fig. 4. It covers many of the islets but not all. Those remaining uncovered expose their receptor sites proper to trap T4-particles when they come near. T2- and T6-particles are caught by different and probably more numerous sites in the lipoprotein. The enzyme of all three phage types removes the bridges in the near vicinity of the point of attachment of each individual particle (Koch & Weidel, 1956), thus opening a thoroughfare for the phage particle's DNA to be injected into the cell, which under ordinary conditions rapidly closes the hole again. Only under exceptional conditions when too many bridges are removed by too many adsorbed phage particles and/or when a repair is made impossible, the inside pressure of the cell will rest directly upon the soft lipoprotein, expanding it into the well-known bubbles which eventually burst. This is called lysis 'from without'. On the other hand, lysis 'from within' at the end of the latent period is very probably caused by a massive attack of free phage-enzyme which, as shown in our laboratory, is produced in surplus in the infected cell (Koch & Jordan, 1957). The surplus is not built into completed phage particles.



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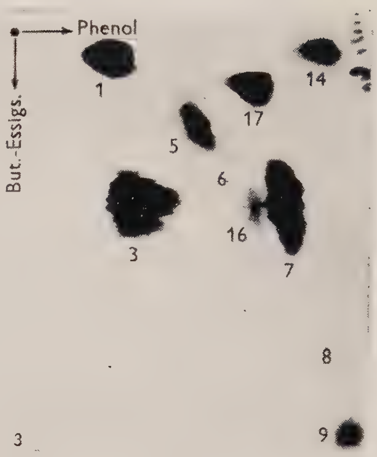

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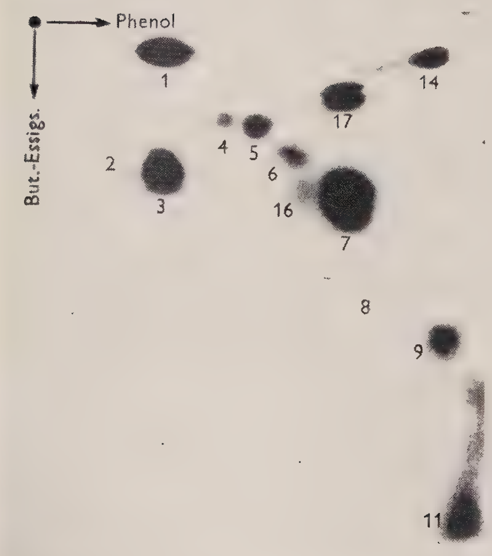

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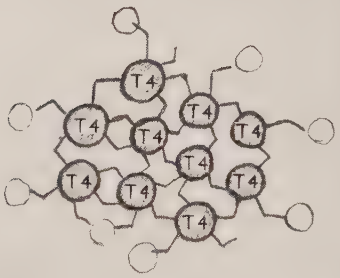
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W. WEIDEL AND J. PRIMOSIGH—BIOCHEMICAL PARALLELS. PLATE 1

(*Facing p.* 517)

The same type of lysis is brought about, in our opinion, where penicillin prevents newly synthesized pieces of cell wall from being protected by a rigid inside harness. Again the whole osmotic pressure is exerted upon the yielding lipoprotein, distorting it into so-called protoplasts, or L-forms, which are actually cells still surrounded by a wall, though an incomplete one. Therefore, as long as the lipoprotein can take up and bear the stress without actually bursting, the cell is safe.

The Gram-negative cell, that is Gram-positive cells which lack the additional protection of an expandable outside layer, are doomed once they are forced by active metabolism in the presence of penicillin to extend their habitually naked walls with pieces of new fabric which are easily disruptible. The comparatively high sensitivity to penicillin which is typical of many Gram-positive bacteria, can thus be accounted for.

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EXPLANATION OF PLATE

Fig. 1. Chromatogram of hydrolysate of lipoprotein layer: 2, asp.; 3, glut.; 4, ser.; 5, gly.; 6, threo.; 7, ala.; 8, tyro.; 9, val. + meth.; 10, ph.ala.; 11, leu. + iso-leu.; 12, prol.; 13, hist.; 14, lys.; 15, arg.

Fig. 2. Chromatogram of hydrolysate of lipopolysaccharide layer: 1, diaminopimelic; 16, muramic; 17, glucosamine; others as in Fig. 1.

Fig. 3. Chromatogram of hydrolysate of soluble complexes split from lipopolysaccharide layer by phage enzyme. Numbers as in Figs. 1 and 2.

Fig. 4. Piece of rigid lipopolysaccharide layer of *coli* B-wall, schematically. Compact particles of co-polymerized glucose, heptose and lipid, carrying receptor sites for phage T4, are interlaced by bridges made up from components indicated in Fig. 3. The bridging complexes are removed by phage enzyme.

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The Mechanism of Lysis by Phage Studied with Defective Lysogenic Bacteria

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Introduction

Almost since the discovery of bacteriophage, attempts have been made to analyse the lytic process which allows the infected cell to release the new crop of phage particles. One has thus looked in phage lysates for a substance able to initiate lysis of non-infected cells of the same species. With bacteriophages acting on Gram-positive bacteria, such attempts have frequently been successful (Gratia & Rhodes, 1923; Wollman & Wollman, 1932; Gratia, 1937; Evans, 1934; Wahl & Josse Goichot, 1950; Maxted, 1957). With phages acting on Gram-negative bacteria, the situation appears to be rather complex. Certain strains of phages form, with certain strains of bacteria, plaques which are surrounded by a halo due to the presence of a lysin, a non-diffusible substance produced during phage multiplication (Sertic, 1929*a*, *b*; Sertic & Boulgakov, 1937). Such substances have now been observed in lysates formed by various phage strains (Anderson, 1945; Panijel & Huppert, 1956; Huppert & Panijel, 1956). Their lytic activity can be demonstrated, not on living cells, but on cells first exposed to ultraviolet radiation (Anderson, 1945) or treated with acetone (Panijel & Huppert, 1956). The mechanism by which such substances initiate bacterial lysis is not clear and their role in the processes of lysis by phage has not yet been established. A new possibility of investigating the process of lysis by phage is offered by the study of lysogenic systems.

Defective lysogenic bacteria

Lysogenic bacteria carry and transmit to their progeny the genetic information which allows them to produce bacteriophage in the absence of infection with extrinsic particles. It can be demonstrated that, in such bacteria, the genetic material of the phage is present in a non-infectious form, the prophage, bound to a specific locus of the host genetic material. During the growth of lysogenic cells, the development of the prophage, which results in the production of infectious particles and lysis of the host, occurs only in a small fraction of the population. In some lysogenic systems, however, phage production and bacterial lysis can be induced in the whole of the population by exposing the cultures to the action of u.v. radiation (induction, see Lwoff, 1953; Jacob, 1954).

From lysogenic bacteria, variants can be obtained in which a mutation of the prophage prevents some process necessary for the formation of infectious particles. Strains carrying such mutant prophages are called defective lysogenic strains. They can be isolated either after infection of sensitive bacteria with temperate phages (Jacob, 1950; Lwoff & Siminovitch, 1951; Arber & Kellenberger, 1958) or among the survivors of normal lysogenic bacteria exposed to heavy doses of u.v. radiation (Lederberg & Lederberg, 1953; Appleyard, 1954; Jacob & Wollman, 1956). In cultures of such bacteria no, or very few, phage particles can be detected.

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Genetic analysis of some defective strains of u.v.-inducible *Escherichia coli* K 12 (λ) has shown that the defective character can be ascribed to prophage mutations which interfere with the normal process of phage production (Jacob & Wollman, 1956). After u.v. induction, prophage development is initiated but the prophage lesion results in an alteration of one of the steps necessary for the completion of infectious particles, such as maturation. The rate at which infectious particles are formed results from the back mutation of the defective prophage gene to the wild-type allele. Defective lysogenic bacteria offer therefore the remarkable situation of a phage genetic material that can be perpetuated only in the prophage state. Defective mutations can occur at different loci in the prophage linkage-group and each of these mutations appears to result in an alteration of a different step of phage synthesis and morphogenesis.

When a variety of defective lysogenic strains of *Escherichia coli* K 12 (λ) was examined, it was observed that such strains react differently to u.v. irradiation: some lyse and others do not. Such defective systems therefore provide an opportunity of analysing the process of lysis by phage.

The lysis of defective strains

When exponential cultures of normal lysogenic *Escherichia coli* K 12 (λ) are exposed to suitable doses of u.v. radiation, bacterial growth first proceeds, although at a decreasing rate, during about 70–80 min. (Fig. 1). During this latent period, no infectious particles are released. Lysis then begins and infectious particles are released into the medium. These processes are completed at about 120–150 min.

When defective strains are exposed to the same dose of u.v. radiation and their pattern of lysis examined, three different types of reactions can be observed, depending upon the strain used:

(1) Some of the defective strains behave as normal lysogenic bacteria. After irradiation, residual growth is observed and lysis occurs after a latent period of 80 min. although no infectious particles can be detected in the lysate (Fig. 2, curve 1).

(2) In other strains, residual growth occurs during the latent period, but at 80 min., when growth stops, no lysis occurs and optical density remains constant for several hours (Fig. 2, curve 2).

(3) In a third type of strain, residual growth does not stop at 80 min. but, instead, proceeds for 2–3 hr. The variation in optical density progressively decreases and finally stops (Fig. 2, curve 3).

In u.v.-inducible lysogenic systems, the presence of the prophage confers to the host-bacterium an increased sensitivity to u.v. radiation as compared with

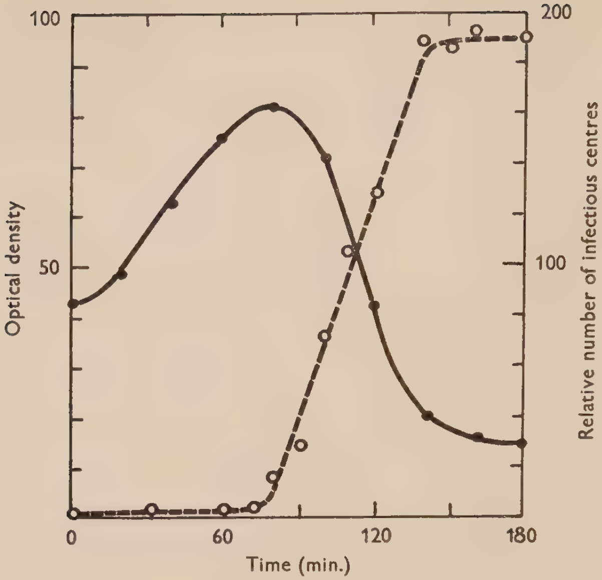

Fig. 1. Ultraviolet-induction of lysogenic *Escherichia coli* K12 (λ). A culture of exponentially-growing bacteria is exposed to an optimal dose of u.v. radiation (inducing phage production in more than 90 % of the population). The suspension is aerated in broth at 37°. The optical density in arbitrary units and the relative number of infectious centres are plotted against the time of incubation after irradiation.

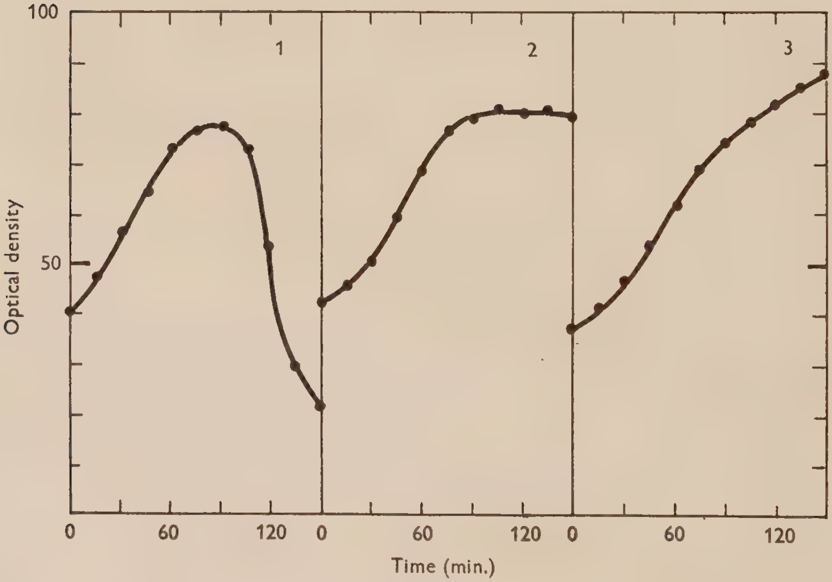

Fig. 2. Different responses of defective lysogenic bacteria to u.v. radiation. Cultures of exponentially-growing bacteria are exposed to an optimal dose of u.v. radiation and aerated in broth at 37°. The optical density is plotted against the time of incubation after irradiation. Curve 1: strain P 32; curve 2, strain P 30; curve 3, strain P 22.

homologous non-lysogenic bacteria. When the sensitivity of u.v. radiation of the various defective types is compared with the sensitivity of normal lysogenic and non-lysogenic bacteria, they are found to be as sensitive as normal lysogenic cells. It appears therefore that u.v. radiation initiates, even in the strains which do not lyse, an abortive development of the prophage which results in an increased sensitivity to u.v. radiation.

The existence of different possible reactions to u.v. radiation, as far as lysis is concerned, indicates that the processes determining bacterial lysis may or may not be initiated in the course of prophage development, according to the type of lesion controlled by the prophage mutation. Comparison of such defective bacteria therefore allows the possibility of investigating the mechanism of lysis in relation with phage multiplication.

The presence of endolysin in the lysates of induced lysogenic bacteria and its role in the process of lysis by phage

The mode of action of lysozyme on bacteria has recently been described in detail (Salton, 1952; Tomcsik & Guex-Holzer, 1952; Weibull, 1953). More especially, it has been found that under defined environmental conditions, lysozyme may also initiate lysis of Gram-negative cells (Repaske, 1956; Zinder & Arndt, 1956). If bacterial lysis resulting from phage action is really due to the presence of a lytic substance, formed during phage development and acting 'from inside' on infected cells, one may wonder whether such a substance would not require the same conditions as lysozyme in order to act 'from outside' on non-infected cells. Indeed, lysates, obtained either after u.v. induction of lysogenic *Escherichia coli* K12(λ) or after infection of sensitive *E. coli* K12 with λ phages, which have been freed from infectious particles by centrifugation, are able to induce lysis of *E. coli* K12, first treated with chloroform or with a salt of ethylene diamine tetra-acetic acid (EDTA) (Fig. 3). If such a lytic activity is really involved in the process of lysis by phage, it must be detected only in the cultures of defective strains which lyse after u.v. irradiation. As a matter of fact, lytic activity can be demonstrated in the lysates of any defective strain which lyses after u.v. irradiation. On the contrary, after ultrasonic disruption of bacteria, a treatment which does not alter the properties of the agent, activity cannot be found, either in growing *E. coli* K12 strains, or in any induced defective strain which does not exhibit lysis after irradiation (Table 1). However, in the latter case, when bacteria are first u.v.-induced and then infected with a normal λ phage, lysis occurs and lytic activity is present in normal amount in the lysates.

It therefore seems reasonable to conclude that bacterial lysis, resulting from the development of phage λ, is correlated with, and probably due to, the presence of a lytic substance which will now be designed as *endolysin*. Formation of endolysin is not restricted to multiplication of phage λ, but appears to be a general feature of phage development since the presence of an endolysin has been found in every phage/bacterium system studied so far, i.e. *Escherichia coli* B-phage T2, *E. coli* B-phage T5, *E. coli* K12-phage 21.

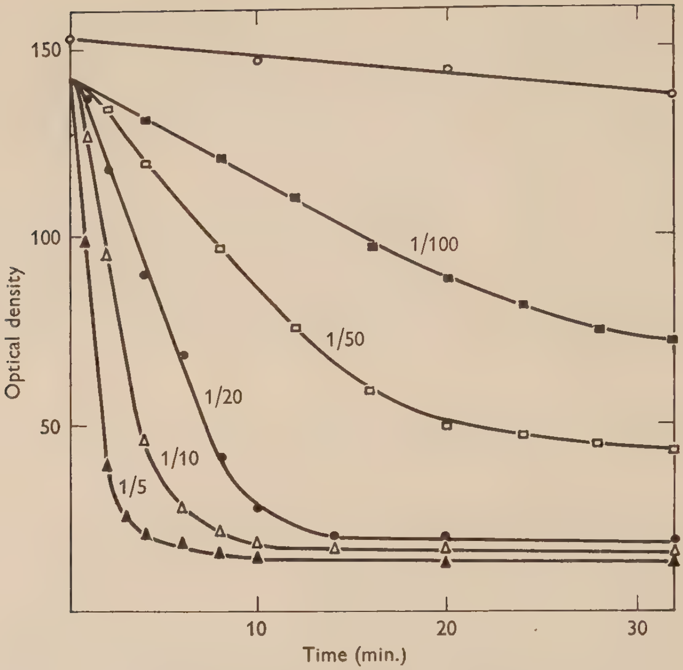

Fig. 3. Action of λ-endolysin on EDTA preparations of *Escherichia coli*. A culture of growing *E. coli* B is centrifuged, washed and the bacteria are suspended in EDTA solution (0·1 M, pH 8·0); 5 min. later the bacteria are centrifuged down, washed and resuspended in distilled water. One ml. of this suspension is added to 1 ml. of various dilutions of a lysate obtained after u.v. induction of defective strain P 32. The mixtures are maintained at room temperature. The optical density of the mixtures, in arbitary units, are plotted against time.

Table 1. *Detection of endolysin in u.v.-induced cultures of various defective lysogenic* Escherichia coli *K* 12

Cultures of various defective strains were exposed to an optimal dose of u.v. radiation, and aerated in broth at 37°. The evolution of the optical density was followed as a function of time. Lysates of strains which lyse, or preparations disrupted ultrasonically of strains which do not lyse were assayed for lytic activity against EDTA preparations of *E. coli* B.

Defective strains	Lysis after u.v. irradiation	Presence of endolysin
P 14	+	+
P 15	+	+
P 16	+	+
P 17	+	+
P 18	+	+
P 19	+	+
P 21	+	+
P 32	+	+
P 34	+	+
P 22	0	0
P 27	0	0
P 28	0	0
P 29	0	0
P 30	0	0

Properties of λ-endolysin

Very little is known as yet about the properties of endolysin: it is not dialysable; the lytic activity is destroyed by heating at 70° for 30 min. or after treatment with trypsin. It appears therefore to be a protein or to contain a protein. The lytic activity is not specific since λ-endolysin is active on EDTA preparations of various strains of *Escherichia coli*, and in particular on strain B which does not adsorb phage λ. It acts also on strains of other species, e.g. *Haemophilus influenzae*. However, this endolysin does not lyse cultures of living Gram-positive bacteria, such as *Bacillus megaterium*, which are lysed by lysozyme.

Like lysozyme, endolysin appears to act on a component of the bacterial cell wall after EDTA treatment, since in the presence of 2 M-sucrose, the cells lose their rod shape and change to small spheres similar to protoplasts. This transformation affects 50–90 % of the population, depending on the preparation. These small spheres are very sensitive to osmotic variations and, like protoplasts, they lyse as soon as they are suspended in distilled water. The chemical nature of the component which behaves as a substrate for endolysin has not yet been analysed.

Endolysin activity may easily be titrated by kinetic measurements of lysis of standard bacterial preparations (Fig. 3). The rate of lysis seems to be roughly proportional to concentration and such a titration appears to be sufficiently accurate for most of the current experiments.

The relationship between endolysin and phage

In many respects, endolysin appears to be similar to, and perhaps is, the same substance as the 'prolysin' found in u.v.-induced lysates of *Escherichia coli* K12(λ) by Huppert & Panijel (1956). By comparing the action of this substance and of trypsin on acetone-treated bacteria, these authors concluded that the lytic substance acted only indirectly by activating autolytic systems of the bacteria. The known properties of endolysin, and especially the possibility to make protoplast-like spheres in the same conditions as with lysozyme, would suggest that endolysin destroys some cell-wall components which have first to be unmasked by a treatment with chloroform or EDTA. Whether or not the autolytic systems of the bacteria play a role in the lytic process still remains unknown.

The question then arises of whether endolysin is synthesized *de novo* during phage multiplication, or whether it exists in the cell in an inactive form and is then unmasked during phage development. After u.v. radiation, the lytic activity may be detected at about 35–40 min., i.e. in the middle of the latent period and then it increases until lysis (Fig. 4). When KCN or chloroamphenicol are added at 40 min., no further increase in the lytic activity is observed (Fig. 4). Although not conclusive, this result would suggest that one is dealing here with the *de novo* synthesis of a protein.

Another point raised by the study of endolysin is the relationship which may exist between the phage produced and endolysin. Is endolysin a phage constituent and, if this be the case, is it related with other phage constituents which are known to exert an enzymic activity on components of susceptible cells (Barrington & Kozloff, 1956; Weidel & Primosigh, 1958)? Or, on the contrary, does endolysin represent some kind of by-product of phage synthesis?

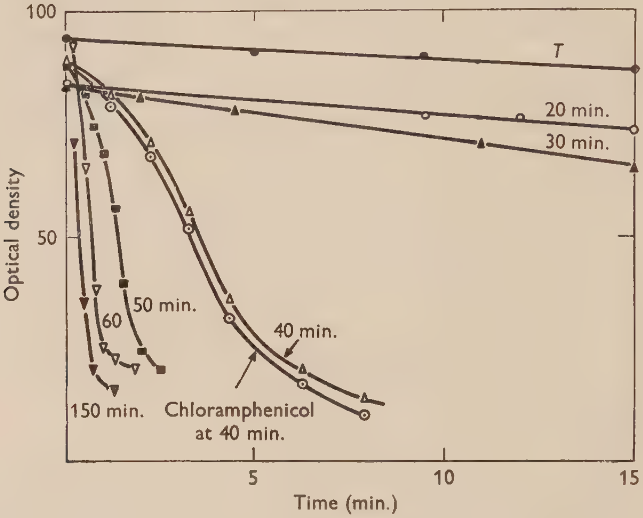

Fig. 4. Variations of lytic activity in u.v.-induced lysogenic cells during the latent period. A culture of defective lysogenic bacteria P 32 is induced at time 0 with an optimal dose of u.v. radiation and the organisms aerated in broth at 37°. At various times, a sample is cooled and the cells are disrupted ultrasonically with a 'Raytheon'. The extracts are assayed on EDTA preparation of *Escherichia coli* B as described in Fig. 3. At 40 min., chloramphenicol (20 μg./ml.) was added to a sample which was aerated at 37° during 40 min. Bacteria were then disrupted ultrasonically and the activity assayed. The optical density of the assay mixtures, in arbitrary units, is plotted against time. Figures represent the times at which samples were cooled.

Satisfactory answers to these questions have not yet been obtained. For the time being, one may mention that anti-λ serum does not exhibit any activity against endolysin and that purified phage particles do not exhibit measurable lytic activity under the conditions in which endolysin acts. If endolysin is a phage constituent, it must therefore be situated inside the external coat of the particle, and lytic activity might only be recovered from infectious particles which had been disrupted.

Summary and conclusions

In lysates resulting from the development of phage λ, a substance, endolysin, may be detected which lyses Gram-negative bacteria in the same conditions as lysozyme does, i.e. after treatment with chloroform or EDTA. Endolysin seems to be a protein. It acts on a component of the bacterial cell wall and, in the presence of 2 M-sucrose, transforms the cells into protoplast-like spheres which are very sensitive to osmotic variations. The lytic activity is not specific since endolysin is active on various strains belonging to different species. Similar substances have been detected in the lysates resulting from the development of the different phages studied so far.

Evidence that endolysin plays a role in the process of lysis by phage results from a comparative study of defective lysogenic bacteria. Endolysin is found in the lysate of every defective strain which does lyse after u.v. radiation. On the contrary, no endolytic activity has been detected in extracts of any strain which does not lyse after irradiation.

During phage development, endolytic activity appears in the middle of the latent period and increases until lysis. The possible nature of endolysin as well as its relationship with phage constituents are discussed.

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Colicins, Colicinogenic Factors and their Relations to Bacteriophages

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Many enteric bacteria, mostly *Escherichia coli* and *Shigella sonnei*, but also other *Shigella* spp. and some *Salmonella* spp., produce a variety of diffusible antibiotic substances, named colicins. All colicins appear to be of a protein nature but differ in many other properties: extent and specificity of their activity spectra, specificity of resistant mutants, diffusibility, morphology of the inhibition zones in agar, susceptibility to proteolytic enzymes, thermoresistance, electrophoretic motility, antigenic properties. Their activity spectra are strikingly different but strictly limited to other strains of the Enterobacteriaceae family. Susceptibility-patterns are controlled by a number of receptors specific of each colicin to which a strain is susceptible. Susceptible bacteria are rapidly killed by the colicin which they adsorb.

The presence of the colicin-receptors is governed by genetic factors which may recombine by crossing or get lost by mutation. These mutations are spontaneous and independent of the other properties. They are specific of each receptor, since a mutant resistant to a given colicin is still inhibited by other colicins acting on the mother-strain. It is, however, possible to transform a strain susceptible to many colicins into a completely resistant one if all its specific receptors are removed successively in a series of mutations.

Colicins display unexpected relations with bacteriophages. Colicinogenic strains of bacteria resemble lysogenic strains, and colicins behave, in many respects, like bacteriophages. Activity spectra of both agents are quite comparable in their diversity, and susceptibility-patterns are controlled in each case by a number of specific receptors, some of which may be common to a phage and a given colicin. The bactericidal activity of colicins is comparable to that of virulent bacteriophages, more particularly to their killing action without multiplication of the particles. In fact, the bactericidal components of some virulent bacteriophages must be substances related to colicins. For example, the bactericidal component of coliphage T6 is a protein which has the same specificity as colicin K, adsorbs on the same receptor and displays the same sensitivity to X-ray inactivation.

Colicins and bacteriophages are, however, agents of a fundamentally different nature. Colicins are chemical substances, proteins, which kill bacteria without being reproduced, whereas bacteriophages are biological units, endowed with genetic continuity, which are reproduced and multiplied in the cells they kill. Bacteriophages are much more complex particles which include a deoxyribonucleic core and a protein envelope.

The genetic structure which is included in the phage particles and induce their reproduction is absent from colicins. Owing to the hereditary stability and specificity of the colicinogenic properties, such genetic structures must, however, be present in the bacteria which produce the colicins. These genetic factors can be demonstrated by the fact that they may be transmitted from one strain to another. As they are not set free by lysis of the cells, their transfer requires the mechanism of sexual conjugation, but they behave, in recombination experiments, as if completely independent of the normal genetic structure of the bacteria.

Like prophages in lysogenic strains, colicinogenic factors are potential lethal agents whose spontaneous or induced development kills the cells in which it occurs. They are also pathogenic, although their pathogenicity is only disclosed by the achievement of their potentiality. As long as they remain in a latent condition, they induce, on the other hand, immunity to the corresponding colicin. Colicinogenic factors are transmissible pathogenic agents which are independent of the normal genetic structure of bacteria and might therefore be regarded as bacterial viruses, distinct from bacteriophages. Their relations with the latter may perhaps point to a common parental ancestry.

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Lysis of Gram-Negative Bacteria by Serum

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The antibacterial property of serum, first reported by Nuttall in 1888, has attracted many investigators and an extensive literature has accumulated. The sera of most mammals tested have been found to possess bactericidal activity, but a notable exception is the mouse, whose serum, apparently through lack of complement, is unable to kill any of a number of bacterial strains which are sensitive to other sera (Marcus, Esplin & Donaldson, 1954). Many types of bacteria are sensitive to serum killing, although definite lysis does not invariably occur. Most workers, including ourselves, have been concerned primarily with killing, as measured by failure to produce colonies on a culture plate.

Table 1. *Inactivation of the bactericidal power of human serum by inactivation of the components of complement*

Treatment of serum	Complement components inactivated
Heat at 56° for 30 min.	C′1, C′2, C′3
Absorption with antigen-antibody precipitate	C′2, C′4
Zymosan at 37°	C′3
NH_4OH	C′4

Several distinct bactericidal mechanisms in serum have been described. It is widely accepted that Gram-negative organisms in general are killed by mechanisms which involve the thermolabile agent complement. Complement itself is not a single entity but consists of four components designated C′1, C′2, C′3 and C′4, all of which are necessary for bactericidal activity, and all of which are believed to be proteins. None of the components of complement has been isolated in the pure state, although highly enriched preparations were obtained by Pillemer, Ecker, Oncley & Cohn (1941) and by Lepow, Ratnoff, Rosen & Pillemer (1956). Almost all the studies on complement components have hitherto been done, not with preparations of individual components, but with serum lacking one or more components as a result of various treatments (Table 1). Different opinions have been expressed about the ability of complement itself to kill bacteria or whether antibody is invariably required. Different views have also been held about the origin, specificity and role of the naturally occurring antibodies. It is not, however, our purpose to review this topic here as it has been discussed in standard texts (e.g. *Topley and Wilson's Principles*, 1955).

Investigation has shown that the complement-dependent bactericidal activity of normal human serum towards certain organisms requires magnesium ion, and also a serum euglobulin, properdin, which is distinct from antibody and complement (Pillemer, Blum, Lepow, Ross, Todd & Wardlaw, 1954; Wardlaw & Pillemer, 1956). The requirement for properdin was demonstrated by the loss of bactericidal activity following absorption of serum with the yeast cell-wall fraction 'zymosan'. The conditions of absorption were controlled so that properdin was removed without loss of component C′3. Properdin itself was eluted from the zymosan residue, and was able to restore bactericidal activity to properdin-deficient serum. Properdin by itself was non-bactericidal. The requirement for Mg ion was similarly demonstrated by the decrease in bactericidal activity following treatment of the serum with the cation exchange resin Amberlite IRC-50 (sodium cycle). Addition of $MgCl_2$ restored bactericidal activity to resin-treated serum. It was found with the same test organism (*Shigella shigae*) that removal of any of the four components of complement from serum also resulted in loss of bactericidal activity. Thus, altogether at least six distinct serum factors participate in the destruction of this organism. Clearly an extremely complex sequence of reactions remains to be defined.

Table 2. *The greatest dilution of various sera which produced 50 % killing of a standard inoculum in 90 min.*

Strain	Guinea pig	Rat	Human	Rabbit	Chicken
Escherichia coli 2206	1/2	1/2	1/20	1/32	1/32
E. coli K12	1/1	1/16	1/100	1/16	1/4

A possible approach to this problem is suggested by several procedures which, when applied to bacteria, alter their serum sensitivity. A well-authenticated example is that rough mutants of a given strain are usually much more sensitive to serum than the smooth parent, which suggests that the somatic (O) antigen may in some way be involved. Another example is the finding that bacteria in the logarithmic phase of growth are much more readily killed by serum than those in the lag phase (Fig. 1). Thus, in the experiment with *Escherichia coli* illustrated, bacteria in the logarithmic phase were killed in less than 5 min., whereas lag-phase organisms were still viable at 60 min. The sensitivity of bacteria can also be altered by the choice of culture medium. Thus, organisms grown in broth containing 0·005 M-phloridzin were much more sensitive than organisms grown in broth without phloridzin (Fig. 2).

Another type of variation that is frequently encountered is the variation in bactericidal activity of different sera and the sensitivity of various strains (Table 2). It is difficult to decide from these data which is the most sensitive strain and which is the most active serum. *Escherichia coli* 2206 is less sensitive to rat serum than *E. coli* K12, yet the reverse is true for chicken serum; human serum is the most active against strain K12. These variations make difficult the comparison of the results obtained by different investigators who have used a wide variety of test organisms and sera.

With regard to the mechanism of bactericidal action, we have considered the hypothesis that serum contains an enzyme system whose substrate is an essential component of the bacterial cell. The finding that *Escherichia coli* grown in the presence of phloridzin, which is a known inhibitor of phosphorylation, had a lowered resistance, suggested that a phosphate-containing structure might be attacked. The knowledge that smooth strains were more resistant than their rough mutants led to a consideration of the somatic (O) antigen as a possible substrate. On this basis, the lipopolysaccharide component of this antigen has been labelled with ^{32}P as an integral part of the macromolecule (Rowley, Howard & Jenkin, 1956). These lipopolysaccharides have molecular weights in the range of 1–10 million and are therefore non-diffusible through a cellophane membrane. The action of serum on these materials was examined by incubating mixtures of serum and ^{32}P-lipopolysaccharide in tubes for 30 min., then coolling to 0°, transferring the contents to a dialyser and dialysing overnight at 2° against saline. The diffusate was counted for radioactivity and the proportion of ^{32}P liberated from lipopolysaccharide then calculated. With this technique it was found that serum liberated up to 25 % of the total ^{32}P of the lipopolysaccharide in the form of inorganic phosphate. The reaction appeared to be enzymic in that it had temperature and pH optima of 37° and 8·5 respectively and was inhibited by EDTA. Heating the serum at 56° for 30 min. completely destroyed activity. Whole haemolytic complement was not required for the reaction, since mouse serum was quite active, as was human serum from which complement component C′3 had been removed. These data

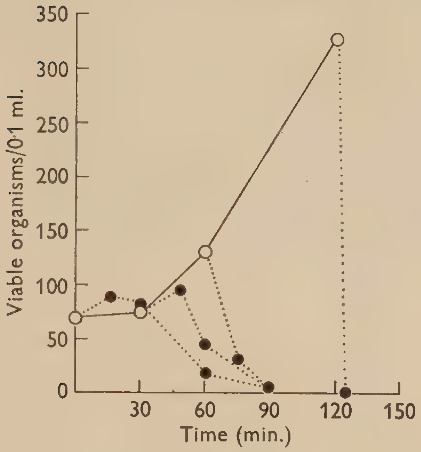

Fig. 1

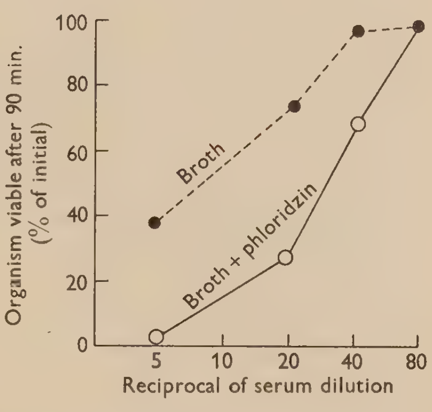

Fig. 2

Fig. 1. Changes in sensitivity of *Escherichia coli* to bactericidal action of serum at different phases in the growth cycle. The solid line shows the increase in viable count following inoculation of the organisms into heat-inactivated serum and incubation at 37°. The dotted lines are death-time curves of samples taken from this culture and immediately transferred to unheated serum.

Fig. 2. Bactericidal activity of dilutions of human serum on *Escherichia coli* grown in broth, with and without added phloridzin.

identify the enzyme as an alkaline phosphatase, and this is confirmed by the finding that purified intestinal alkaline phosphatase liberated the same 25 % of ^{32}P at a similar rate. Other data indicate that the serum enzyme which splits phosphate from lipopolysaccharide is not identical with the serum alkaline phosphatase that is measured by the King-Armstrong method (hydrolysis of phenol phosphate). Thus, the two activities did not run parallel in different sera and serum fractions. Furthermore, phenol phosphate did not inhibit the action of serum on lipopolysaccharide, whereas lipopolysaccharide strongly inhibited the action of serum on phenol phosphate. It seems, therefore, that serum may contain two phosphatases, one of low specificity capable of acting on phenol phosphate and lipopolysaccharide the other of high specificity and capable of acting only on lipopolysaccharide. As yet there is no definite evidence that the dephosphorylating action of serum on lipopolysaccharide is in any way involved in the bactericidal action of serum on living cells. However, certain data suggest that there may be a connexion between the two phenomena (Table 3). Here a comparison is made between the sensitivities of various lipopolysaccharides to the dephosphorylating action of serum and the serum sensitivities of the parent strains. It is apparent that in general the most sensitive strains yielded the most sensitive lipopolysaccharides and vice versa.

Table 3. *Effect of rat serum on various bacterial strains and the radioactive lipopolysaccharides isolated from them*

Organism	Reciprocal of serum dilution giving 50 % maximum splitting of ^{32}P-lipopolysaccharide	Reciprocal of serum dilution giving 50 % killing of parent strain in 90 min.
Salmonella paratyphi B11 (rough)	1000	50
Escherichia coli (Lilley)	200	40
S. paratyphi B1 (rough)	200	50
E. coli 2206	100	10
S. paratyphi B1 (smooth)	25	4
S. paratyphi B11 (smooth)	25	4
E. coli 2380	20	1
E. coli 145	10	>1

If, in conclusion, one compares the present state of knowledge of serum bactericidal action with that existing around 1900, it is apparent that we yet know little more about the actual mechanism of the reaction. Furthermore, the discovery of new serum factors without accompanying knowledge of their modes of action greatly complicates the task. It is clear from other papers given in this Discussion that hypotheses other than the one we have held will have to be considered. But even if one adheres to the enzyme-substrate type of hypothesis, there is still a basic theoretical question to settle, and this applies equally to the lysozyme system, namely, if one takes bacterial strains of increasing resistance to serum, is their resistance due to a greater or lesser content of substrate or to an altered substrate, or what? It is apparent that theoretical arguments can be advanced in favour of any of these.

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The Extent of Acetate and Ethanol Oxidation by *Euglena gracilis*

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SUMMARY: *Euglena gracilis* utilizes acetate and ethanol as energy and carbon sources. The O_2 consumption due to acetate was equivalent to a 42 % oxidation of the available acetate. CO_2 production in the presence of acetate was consistent with the assumption that acetate is simultaneously oxidized and converted to carbohydrate.

Addition of ethanol resulted in an O_2 consumption equivalent to a 39 % oxidation of this substrate. The CO_2 production due to ethanol was 0·93 μmole per μmole of ethanol, more than double the amount predicted if carbohydrate is the sole synthetic product of ethanol metabolism.

Conditions known to change the rate of respiration, such as composition of growth media, incubation pH, and the adaptation to acetate of ethanol-grown cells, had no effect upon the extent of ethanol and acetate oxidation.

Euglena gracilis is a member of a physiological group of protozoa termed the ‘acetate flagellates’, characterized by their excellent growth with acetate as the sole carbon source, and their poor utilization of glucose and related compounds (Hutner & Provasoli, 1951). Until recently, the mechanisms regulating the oxidative metabolism of the acetate flagellates had been little investigated.

Lewin (1954) described adaptive changes in the oxidation: assimilation ratio of acetate in *Chlamydomonas dysosmos*. Danforth & Wilson (1957) reported that Euglena, when grown upon acetate as a carbon source at pH 7·3 (Ac-grown), oxidized acetate with a higher and less pH-sensitive rate than did cells grown upon ethanol as a carbon source at pH 3·5 (Et-grown). In addition, some Et-grown cultures adapted to acetate, acquiring rates of acetate oxidation characteristic of Ac-grown cells.

A study of the extent of acetate and ethanol oxidation was undertaken for the purpose of obtaining basic information concerning the relationships of the energetic and synthetic activities of Euglena, and to ascertain whether conditions known to vary the rate of acetate respiration would also vary the extent of acetate oxidation.

METHODS

A streptomycin-bleached (non-photosynthetic) strain of *Euglena gracilis* var. *bacillaris*, the same as used in previous studies, was maintained in 10 ml. stock tubes on an acetate + tryptone medium at pH 7·3. Experimental cultures were grown under the two conditions listed in Table 1; Et-grown cells were grown on an ethanol + tryptone medium adjusted to pH 3·5; Ac-grown cells were grown on the above-mentioned acetate + tryptone medium at pH 7·3.

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Details of the growth procedures have been published elsewhere (Danforth & Wilson, 1957).

All cultures were incubated at 18–23° until visual inspection indicated good growth, usually within 7–14 days.

Table 1. *Composition of growth media*

	Ac-grown	Et-grown
Bacto-tryptone (Difco)	5 g./l.	5 g./l.
Na acetate	18·4 mM	0
Ethanol	0	18·4 mM
pH	7·3	3·5

The cells were then harvested and washed by centrifugation, and suspended in distilled water. A sample of this suspension was removed, fixed with ethanolic $HgCl_2$, and counted in a haemocytometer.

Experimental solutions of Na acetate.$3H_2O$ and ethanol ranged from 0·8 to 120 mM; only the acetate solutions were adjusted for pH. The buffer for all determinations was an equimolar mixture of tris(hydroxy-methyl)amino-methane and phosphate yielding a final concentration of 0·011 M. Experiments were run at pH 5·5 or pH 7·3.

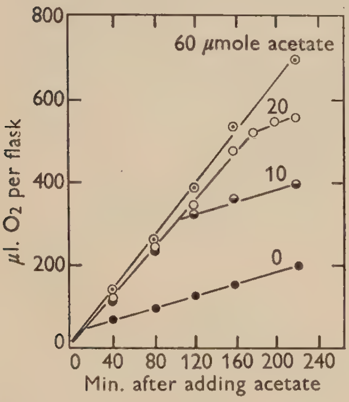
Determinations were conducted with a Warburg apparatus at 26·1°, having a shake rate of 120/min. and a gas phase of air. O_2 consumption was measured with 0·1 ml. of 14% NaOH or KOH in the centre well. CO_2 production was measured by the direct method (Umbreit, Burris & Stauffer, 1949) replacing the alkali in the centre well with water. The time course of CO_2 production was measured at pH 5·5, while at pH 7·3, acid was tipped into the flasks, before and at the completion of an experiment, to ascertain the total amount of CO_2 evolved.

To determine the extent of acetate and ethanol oxidation cells and buffer were added to the main compartment and 2–20 μmole substrate in 0·5 ml. were added to the side arm. The substrate was tipped into the main compartment after at least a 20 min. equilibration period, and the O_2 consumption was recorded until the respiration had returned to the endogenous level. The total amount of O_2 consumed due to the added substrate was calculated by subtracting the O_2 consumed by an endogenous control during the course of the experiment.

These values were then plotted against the amounts of substrates added, and the slope of a line of best fit, using the Least Squares method, gave a value for the average O_2 consumption per μmole of substrate. The percentage of the substrate oxidized was calculated by dividing the average O_2 consumption by the O_2 consumption expected if the substrate were 100% oxidized ($3O_2$:ethanol, $2O_2$:acetate).

RESULTS

Euglena has a low, but measurable, rate of respiration when incubated in the absence of substrate. The respiration rate increases and remains constant for some time when an oxidizable substrate is added to the cells. After the respiration rate returns to the endogenous level, all the substrate is assumed to be removed from the medium. Figure 1 illustrates this behaviour in the case of Ac-grown cells, incubated with varying amounts of acetate.



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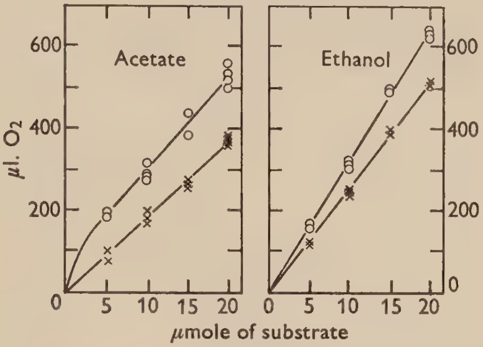

Fig. 2

Fig. 1. O_2 consumption of Ac-grown cells on acetate. 0, 10, 20, 60 μmole of acetate added at zero time. Cells incubated at pH 5·5.

Fig. 2. Relationship between total O_2 consumption of Euglena and amount of substrate added. ○, assuming endogenous respiration suppressed by substrate; ×, assuming endogenous respiration continues during substrate oxidation. Data for acetate include 13 determinations from 4 separate experiments; for ethanol, 11 determinations from 3 experiments.

In order to calculate the efficiency of substrate metabolism by Euglena, it is necessary to determine whether the endogenous metabolism is inhibited during substrate oxidation, as has been reported for other organisms (Barker, 1936; Syrett, 1951). If the endogenous metabolism is suppressed by the presence of exogenous substrate, all the oxygen consumed during the presence of substrate must be attributed to substrate oxidation, while if the endogenous metabolism continues unchanged, the endogenous oxygen consumption must be subtracted from the total consumption in order to determine the oxygen consumption due to the substrate. Figure 2 presents data bearing on this problem. In this figure the same data are plotted in two ways: first on the assumption that the endogenous respiration is suppressed in the presence of substrate, and also on the assumption that the endogenous respiration continues unchanged. In the case of ethanol oxidation, both assumptions result in a constant ratio of oxygen consumption to substrate, while for acetate only the assumption that endogenous respiration continues yields such a constant ratio.

As Syrett (1951) has pointed out, under certain conditions both assumptions concerning the behaviour of the endogenous respiration in the presence of substrate may produce a constant ratio of oxygen consumption:substrate consumption, and in such cases, this criterion is of no value in distinguishing between the two alternatives. This is clearly the case for ethanol oxidation by Euglena. In the case of acetate, on the other hand, only the assumption that endogenous respiration continues during substrate oxidation gives a constant oxygen : substrate ratio.

It is concluded, therefore, that endogenous metabolism continues unchanged during oxidation of exogenous acetate. In the case of ethanol, no direct evidence supports such a conclusion, but since it seems likely that the two substrates would have similar effects upon the endogenous metabolism, it is assumed that neither acetate nor ethanol suppresses the endogenous metabolism.

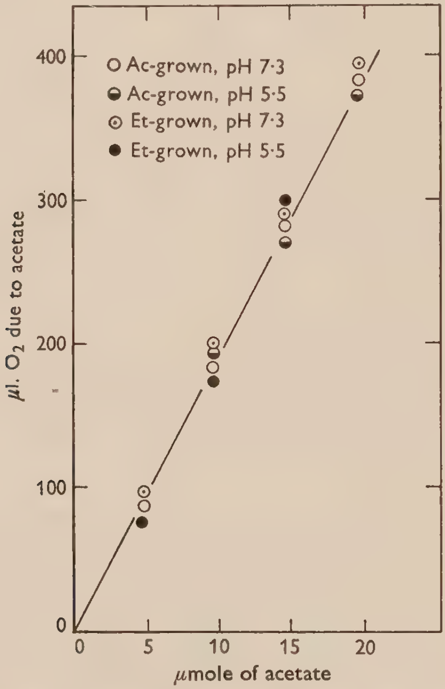

Fig. 3. The extent of acetate oxidation by Euglena. Values of a single class (e.g. Ac-grown cells at pH 7·3 with 10 μmole of acetate) averaged together. Slope is 18·8 μl. O_2 : μmole acetate, equivalent to a 42 % oxidation of acetate.

In Fig. 3, the O_2 consumption due to acetate, in a series of 44 trials, is plotted against the initial amounts of acetate added. Experiments were conducted with Et-grown and Ac-grown cells, incubated at pH 5·5 and 7·3. Cell concentrations ranged from $1{\cdot}5 \times 10^6$ to 10×10^6 cells per flask. Acetate amounts ranged from 0·8 to 20 μmole. The slope of the line of best fit is 18·8 μl. O_2 per μmole of acetate added, and is equivalent to the oxidation of 42 % of the available acetate.

Adaptation to acetate had no effect upon the extent of acetate oxidation. Figure 4 illustrates the events during the adaptation to acetate of cells from an 8-day-old culture of Et-grown cells. The rate of acetate respiration increased 44 %, but the extent of acetate oxidation was no different from that found in the absence of adaptation.

The time course of CO_2 production, determined at pH 5·5, was linear (except during adaptation), and the respiratory quotient of acetate oxidation was 1·01 ± 0·08, independent of the experimental conditions. The results of 15 trials, at pH 5·5 and 7·3, of Ac-grown and Et-grown cells, are tabulated in Table 2. Adaptation to acetate, which occurred in 7 of the 15 trials, had no effect upon the respiratory quotient.

Table 2. *The respiratory quotient of Euglena on acetate*

Growth substrate	Acetate (mM)	Respiratory quotient pH 5·5	pH 7·3
Ethanol	30	1·00	—
	30*	1·10	—
	10*	1·06†	—
	30*	0·75	1·06†
	30*	—	0·90†
	30*	—	0·96†
	30	0·93†	0·91†
Acetate	30	1·00	—
	30	1·17	1·35†
	30	1·06†	1·04†
Average		1·01	1·04
Average (all values)		1·02 ± 0·09‡	(9 %)

* Adaptation. † Acid-tipped method. ‡ Average deviation.

The extent of ethanol oxidation was determined at pH 5·5 and 7·3, using Et-grown and Ac-grown cells. Cell concentrations ranged from $2{\cdot}0 \times 10^6$ to $7{\cdot}0 \times 10^6$ cells per flask, and the initial amounts of ethanol ranged from 2·5 to 30 μmole. In Fig. 5, the O_2 consumption due to ethanol is plotted against the initial amounts of ethanol. The slope of the line of best fit is equivalent to a 39 % oxidation of the ethanol added to the cells.

The time course of CO_2 production, in the presence of ethanol at pH 5·5, was linear with both Et-grown and Ac-grown Euglena. The results of 12 trials, at low and high pH, using Et-grown and Ac-grown cells, presented in Table 3, yield 0·33 ± 0·04 as the respiratory quotient for ethanol oxidation, independent of the experimental conditions.

Since there is no direct evidence that the endogenous metabolism continues during the oxidation of ethanol, the data for ethanol oxidation have also been calculated on the assumption that endogenous respiration is suppressed in the presence of exogenous ethanol. Under this hypothesis, the pertinent values become: μl. O_2 per μmole ethanol, 32·5 ± 1·2; respiratory quotient on ethanol, 0·40 ± 0·004.

DISCUSSION

There is a certain circularity in concluding that the endogenous metabolism continues during acetate oxidation because this assumption gives constant oxygen:substrate ratios and then using data corrected on the basis of this conclusion to demonstrate that oxygen:acetate ratios do not change with

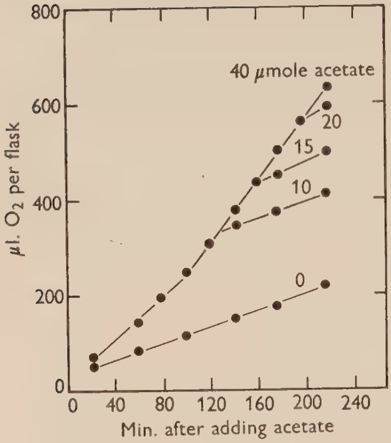

Fig. 4

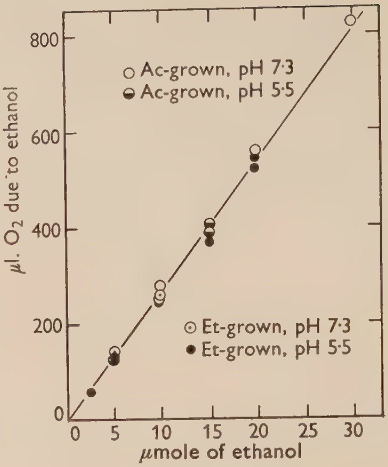

Fig. 5

Fig. 4. Effect of adaptation to acetate on extent of acetate oxidation. Et-grown cells, incubated at pH 7·3 with 0, 10, 15, 20, 40 μmole of acetate. Average μl. O_2 due to acetate: μmole acetate (for 10, 15, 20 μmole acetate) is 18·8.

Fig. 5. The extent of ethanol oxidation by Euglena. Slope is 26·3 μl. O_2: μmole ethanol, equivalent to a 39 % oxidation of ethanol.

Table 3. *The respiratory quotient of Euglena on ethanol*

Growth substrate	Ethanol (mM)	Respiratory quotient pH 5·5	Respiratory quotient pH 7·3
Ethanol	30	0·29*	0·23*
	30	0·41	—
	5	0·28	—
	30	—	0·32
	30	—	0·32
Acetate	3	0·31	—
	30	0·34	—
	30	0·34*	0·40*
	30	—	0·34
	30	—	0·37
Average		0·33	0·33
Average (all values)		0·33 ± 0·04†	(12 %)

* Acid-tipped method. † Average deviation.

environmental conditions. In our opinion, recognition of this circularity does not seriously weaken the conclusions. If the extent of oxidation of acetate actually varied, there is no reason to believe that any simple assumption concerning the endogenous respiration should give constant ratios. Moreover, it is apparent from Fig. 2 that the variation in oxygen: substrate ratios introduced by using the 'wrong' assumption concerning the endogenous respiration is chiefly between results obtained with different amounts of acetate. Similar results in this respect were obtained regardless of the past history of the cells or the pH of incubation. Such variations are irrelevant to the conclusion that neither growth medium nor pH alters the efficiency of acetate metabolism by Euglena.

Euglena incubated on acetate consumed 0·84 μmole of O_2 for each μmole of acetate utilized, equivalent to a 42 % oxidation of this substrate, independent of the experimental conditions. Similar values have been reported for other organisms. Barker (1936) observed at 50 % oxidation of acetate with *Prototheca zopfii*, a colourless alga. Using a differential calorimetric technique Blum, Podolsky & Hutchens (1951) found that *Chilomonas paramecium* oxidized 40 % of the available acetate. Cirillo's data (1956) indicate that *Polytoma uvella*, a phytoflagellate, oxidized 46 % of the available acetate.

Assuming that all the acetate not oxidized was assimilated by the cells, the oxidation: assimilation ratio of acetate was 0·72 for Euglena. The respiratory quotient of acetate was 1·0, independent of the experimental conditions; 0·84 μmole of CO_2 was produced for each μmole of acetate utilized. If all the acetate not oxidized were converted into a carbohydrate-like material, the empirical equation for the process would then be:

$$CH_3COOH + 0{\cdot}84\ O_2 \rightarrow 0{\cdot}84\ CO_2 + 0{\cdot}84\ H_2O + 1{\cdot}16\ CH_2O.$$

This equation satisfies the theoretical conditions for the simultaneous oxidation of acetate and its conversion to carbohydrate, and the respiratory quotient and percentage oxidation experimentally observed. The results are thus consistent with the simultaneous oxidation and conversion to carbohydrate of acetate, but cannot be considered proof that such is the case.

Addition of ethanol to Euglena resulted in the consumption of 1·17 μmole of O_2 for each μmole of ethanol utilized, equivalent to the complete oxidation of 39 % of this substrate, independent of the experimental conditions. Again assuming that all the substrate not oxidized was assimilated by the cells, the oxidation: assimilation ratio of ethanol was 0·64. If all the ethanol not oxidized were converted into a carbohydrate-like material, the theoretical equation for this simultaneous oxidation and synthesis may be represented as (based on the O_2: ethanol ratios)

$$C_2H_5OH + 1{\cdot}17\ O_2 \rightarrow 0{\cdot}17\ CO_2 + 1{\cdot}17\ H_2O + 1{\cdot}83\ CH_2O.$$

The respiratory quotient predicted by this equation is 0·15, while the experimentally determined respiratory quotient for ethanol oxidation was 0·33. Instead of 0·17 μmole of CO_2 being produced for each μmole of ethanol utilized, it was found that 0·39 μmole of CO_2 was produced. The results do not agree with the assumption that carbohydrate is the sole synthetic product of ethanol metabolism.

Lewin (1954) reported an adaptation of *Chlamydomonas dysosmos* apparently involving their ability to assimilate acetate. The cells oxidized acetate at once, but a 60 min. exposure period was necessary before assimilation began.

Although it is not pointed out in the text, the data of Tanenbaum (1956, figs. 1, 2) indicate that ethanol-grown Acetobacter also adapt to acetate. O_2 consumption and CO_2 production due to acetate reached maximum rates only after a lag of about 10 min. On ethanol, CO_2 production was similarly delayed, but O_2 consumption began immediately at its maximum rate. This suggests that, in this case, ethanol is promptly oxidized to the level of acetate, but acetate cannot be oxidized without a brief adaptation period. In Euglena, the extent of oxidation of both ethanol and acetate was constant under all experimental conditions. There is evidence (Danforth & Wilson, 1957) that the adaptive change in the rate of acetate metabolism of Euglena involves an increased permeability to acetate ions. Thus, it would appear that, in three different micro-organisms which show adaptive changes in acetate metabolism, three distinct adaptive mechanisms are involved.

Conditions markedly affecting the rate of substrate oxidation in Euglena, such as adaptation to acetate, altered pH and lowered substrate concentration, had no effect upon the extent of oxidation. The oxidation: assimilation ratios of these substrates did not vary in response to environmental changes. It is reasonable to conclude that under these experimental conditions, the oxidative metabolism of Euglena is not regulated by changes in the extent to which a substrate is oxidized or incorporated. It is possible that other conditions might alter these oxidation-assimilation ratios. Cirillo (1956) reported a 15% increase in the rate of acetate respiration and the extent of total oxidation in the presence of NH_4Cl by Polytoma. The effect of an exogenous nitrogen source on the extent of acetate and ethanol oxidation has not been studied in Euglena.

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Growth Inhibitors and their Antagonists as Mutagens and Antimutagens in *Escherichia coli*

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SUMMARY: Thirty-five substituted benzimidazoles, benzotriazoles and quinoxalines were tested as growth inhibitors on three mutants of *Escherichia coli*, strain W, having different purine requirements and on the ciliate *Tetrahymena geleii*. A number of the compounds, especially those with a nitro group in the benzene ring, were inhibitory. Both organisms were affected in a similar way by the compounds. Several of these analogues were tested for mutagenic activity at the purine and streptomycin loci in a purine-requiring mutant of *E. coli*. Only inhibitory compounds were found to be mutagenic; non-inhibitory concentrations of one analogue, 4-nitro-6-hydroxybenzimidazole (NHB), were, however, also mutagenic. No specific mutagenesis was demonstrated. NHB was found to increase the rate of mutation as determined by the papilla method. Evidence is presented which eliminates the hypothesis that selection rather than mutagenesis was involved. This analogue did not increase the frequency of mutants in non-dividing cells, and was not incorporated into bacterial DNA. The mechanism of this mutagenesis is thought to include the inhibition of enzymes concerned with DNA synthesis. DNA and RNA and their constituents, purine and pyrimidine precursors and analogues, vitamin B_{12} and other vitamins, were found to have no effect on the inhibition or mutagenesis produced by NHB. Several amino acid combinations and one non-mutagenic analogue, 4-hydroxy-6-nitrobenzotriazole, were, however, found to interfere with mutagenesis by NHB. These compounds did not affect the frequency of spontaneous mutants. The amino acid combination was effective only when present simultaneously with the mutagen and did not act by a selection mechanism. The mechanism of antimutagenesis by amino acids may involve their ability to increase the growth rate and also to modify the inhibited nucleic acid metabolism.

The mutagenesis by 5-nitroquinoxaline, a pterin analogue, was also found to be depressed by amino acids in the same purine-requiring mutant. The annulment of mutagenic activity by amino acids was strain and mutagen specific.

Twenty-five known inhibitors of nucleic acid synthesis and antagonists to inhibition were tested for mutagenic activity in five strains of *Escherichia coli*. Several, including 6-mercaptopurine, were found to be mutagenic; in addition, NHB, 5-nitroquinoxaline and 5-aminouracil were mutagenic in most of the strains tested. The mutagenic activity of 5-aminouracil was not annulled by thymine, thymidine or other nucleic acid components, but the mutagenesis of 6-mercaptopurine was annulled by purine bases and by ribosides.

Novick & Szilard (1951) showed that purine analogues increased the rate of mutation to phage T_5 resistance in *Escherichia coli* B/1,t growing in steady-state conditions in the chemostat. On the basis of these findings and those of Fries & Kihlman (1948) who found caffeine to be mutagenic for *Ophiostoma multiannulatum*, the present investigations on chemical mutagenesis were undertaken, utilizing growth inhibitors, the majority of which are analogues of nucleic acid constituents or are known to interfere with nucleic acid metabolism, and growth-inhibitor antagonists. Both the inhibitors and their antagonists were tested as mutagens and antimutagens in *E. coli*. It was supposed that purine analogues, such as substituted benzimidazoles and benzotriazoles, could act by inhibiting enzymes involved in nucleic acid synthesis so that the wrong kind, arrangement, or proportion of constituents would be built into the deoxyribonucleic acid molecule, or that the analogue itself might be incorporated to form an unnatural or abnormal DNA. Further, substances able to act as antagonists to growth inhibition and nucleic acid constituents might prevent this incorporation or enzyme inhibition and thereby act as antimutagens. Novick & Szilard (1952) also reported the antimutagenic activity of purine ribosides on mutagenesis by caffeine and other purine analogues. Guanosine and adenosine depressed the induced as well as the spontaneous mutation rate. The pyrimidine ribosides were ineffective as were riboside components. The annulment of mutagenesis was effected by low concentrations of the ribosides and was competitive. The present study deals mainly with the effects of 4-nitro-6-hydroxybenzimidazole (NHB), 6-mercaptopurine and 5-aminouracil on mutation.

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METHODS

The organisms used were either *Tetrahymena geleii*, strain H, or various strains of *Escherichia coli* which will be described when relevant. The medium for *T. geleii* was modified after Kidder & Dewey (1949), while a modified Gray & Tatum's medium (minimal medium) (Ryan & Schneider, 1948) was used for *E. coli*.

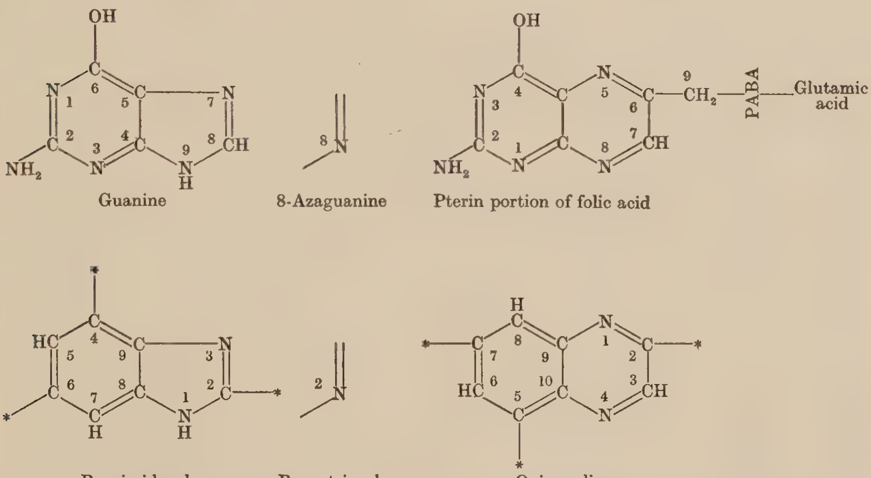
The compounds employed in the inhibition and mutant-screening experiments with *Escherichia coli*, strain W, and its purine-dependent mutants were benzimidazoles, benzotriazoles and quinoxalines, which are structural analogues of purines, the carcinostatic 8-azaguanine and the pterin portion of folic acid (see Fig. 1). These compounds were synthesized by Gillespie, Engelman & Graff (1954, 1956*a*, *b*); see Engelman, Gillespie, Greer & Graff (1952). Strain W and its purine-dependent mutants were obtained from Dr B. Davis (Davis, 1950).

Other compounds were utilized in mutant-screening experiments with *Escherichia coli* mutant strains Wp$^-$, 15h$^-$, 15h$^+$m$^-$, B/r,m$^-$ and B/r,t$^-$. These compounds (shown in Table 7) are inhibitors and their antagonists most of which are analogues of nucleic acid constituents; their effect on metabolism has been studied in other laboratories (Hirschberg, 1955; Woolley, 1951).

RESULTS

Inhibitory and mutagenic activity of benzimidazoles, benzotriazoles and quinoxalines

The effects of the substituted benzimidazoles, benzotriazoles and quinoxalines on the growth of *Escherichia coli*, strain W, its two purine-dependent mutants, and on *Tetrahymena geleii*, strain H, are summarized in Table 1, which also indicates the mutagenic effect of several of the compounds on the guanine-dependent and streptomycin-sensitive loci of mutant Wp⁻. The growth inhibition analyses were performed by inoculating 500 *T. geleii* organisms into 5 ml. medium containing the test compound in test tubes and aerating in a roller at 25°. The growth of *T. geleii* was limited to one-half of maximal at a concentration of 2×10^5 organisms/ml. by adding 6 μg. guanine/ml. In studies with *E. coli*, 5×10^6 organisms of *E. coli*, strain W, or mutants Wp⁻ or Wah⁻, were inoculated into 5 ml. medium containing the test compound in test tubes and aerated on a roller at 37°. The limiting growth factor and sole purine supplement was 6 μg. guanine/ml. which supported one-half of maximal growth in experiments with mutant Wp⁻. In studies with mutant Wah⁻, 18 μg. adenine/ml. served these functions. The limiting growth factor in experiments with wild-type W was 0·1 % (w/v) glucose. For both mutants, several concentrations of the test compounds were used and growth was measured by the increase in optical density as determined in the Klett-Summerson colorimeter. The optical density was calibrated against total and viable bacteria counts. To measure the inhibition of colonial growth, several concentrations of Wp⁻ bacteria (200–1000) were plated in 30 ml. of 1·5 % (w/v) agar containing minimal medium + the test compound and supplemented with 65 μg. guanine/ml. Counts of plates were made after incubation for 10 days at 37°. Determinations of this inhibitory effect of the compounds were made by comparing colony counts with appropriate controls.



Guanine

8-Azaguanine

Pterin portion of folic acid

Benzimidazole

Benzotriazole

Quinoxaline

Fig. 1. A comparison of the structure of benzimidazoles, benzotriazoles and quinoxalines with purines, 8-azaguanine and the pterin portion of folic acid, respectively. * Substitutions of OH, OCH_3, NH_2 and NO_2 groups occur at the 4- and 6-position of benzimidazoles and benzotriazoles and at the 5- and 7-position of quinoxalines. Substitutions of phenyl, ribose, OH and CH_3 groups occur in the 2-position.

The compounds shown in Table 1 have various effects on the micro-organisms. Some affect the length of the lag phase, others also depress the

Table 1. *The effects of substituted benzimidazoles, benzotriazoles and quinoxalines on the growth of* Tetrahymena geleii *and* Escherichia coli *strain W and two of its purine-dependent mutants, including the mutagenic activity of several compounds at the guanine-dependent and streptomycin-sensitive loci of* E. coli *Wp$^-$.*

Organism				*Tetrahymena geleii* strain H	*E. coli* strain W, mutant Wp$^-$ and mutant Wah$^-$*	Effect on survival and mutation of mutant Wp$^-$			
				Concentration of compound 200 μg./ml.				Mean factor of increase over spontaneous mutant-frequency	
Compound. Substitution in position				Degree of inhibition†		Concn. (μg./ml.)	% inhibition of visible colony formation	Purine$^-$ to purine$^+$	Streptomycin^s to streptomycin^r
Benzimidazoles									
2		4	6						
.		.	.	+	+	200	5	2	.
.		OH	NH_2	–	–	.	.	.	.
.		OCH_3	NH_2	+	–	.	.	.	.
.		NH_2	OCH_3	–	–	.	.	.	.
.		.	NO_2	+++++	+++++	5	20	20	4
.		OH	NO_2	+	++	200	98	1	2
.		NO_2	OH	+	+++++	85	64	33	900
						43	17	11	430
						20	0	2	35
						10	0	1	6
OH		NO_2	OH	++++	–	200	0	1	2
CH_3		OH	NO_2	–	+++	200	85	4	1
.		OCH_3	NO_2	++	+	.	.	.	.
OH		OCH_3	NO_2	–	–	100	0	2	1
CH_3		OCH_3	NO_2	++	–	200	0	1	1
ribose		OCH_3	NO_2	+	–	.	.	.	.
.		NO_2	OCH_3	–	–	75	0	1	2
ribose		NO_2	OCH_3	–	–	.	.	.	.
Benzotriazoles									
2		4	6						
.		OH	NH_2	–	–	.	.	.	.
.		OCH_3	NH_2	–	+++	.	.	.	.
.		.	NO_2	+	++++	200	99·9	7	18
.		OH	NO_2	+++	++++	.	.	.	.
.		OCH_3	NO_2	+++++	+++	200	60	8	6
Quinoxalines									
2	3	5	7						
.	.	OCH_3	NH_2	+++++	–	200	0	1	0·2
CH_3	CH_3	OCH_3	NH_2	+	–	.	.	.	.
.	.	NO_2	.	++++	+++++	20	99	14	14
.	.	NO_2	OCH_3	++++	+	200	60	2	2
‡	‡	NO_2	OCH_3	–	–	.	.	.	.
.	.	OCH_3	NO_2	+++++	+++++	40	85	2	1
CH_3	CH_3	OCH_3	NO_2	+++++	++++	200	35	3	3

* Wp$^-$: requiring guanine, adenine, hypoxanthine, xanthine or 4-amino, 5-imidazolecarboxamide, Wah$^-$: requiring adenine or hypoxanthine.

† –, no inhibition; +, time to reach stationary phase = > 1 × control; ++, time to reach stationary phase = > 1·5 × control; +++, time to reach stationary phase = > 2 × control; ++++, time to reach stationary phase = > 2·5 × control; +++++, completely inhibitory to an inoculum of 10^6 bacteria/ml. or 10^4 ciliates/ml.

‡ Phenyl.

rate of growth. In general, the response of the micro-organisms to the compounds was similar. Furthermore, there are no differences in response to these agents by the different nutritional mutants of *Escherichia coli* W. A nitro group on the benzene ring is a highly inhibitory configuration; amino groups are less inhibitory. Adding phenyl, ribose or methyl to the 2-position tends to lessen the inhibitory activity of an analogue.

To determine the frequency of chemically-induced mutants, approximately 10^5 cells of mutant *Escherichia coli* Wp^- were inoculated into flasks containing 100 ml. minimal media supplemented with a superoptimal concentration of guanine (65 μg./ml.) and the test compound. The flasks were aerated on a shaker at 37°. By restricting the glucose to 0·1% (w/v), the growth was limited to 2×10^9 bacteria/ml. after 18 generations. After the bacteria had reached the stationary phase of growth, they were washed twice in 0·85% (w/v) saline (used for all washings and dilutions) and plated. Samples containing 10^{10} bacteria/ml. were plated in Petri dishes containing minimal medium + 1·5% (w/v) agar supplemented with 65 μg. guanine/ml. + 100 μg. streptomycin/ml. After 4 days the number of streptomycin-resistant colonies was determined. Several dilutions of bacteria (no more the 10^9/ml.) were plated in 1·5% minimal agar and the number of guanine-independent colonies determined at 2·5 days. The maximal number of bacteria to be plated in streptomycin agar or minimal agar for full expression of mutants was determined after reconstruction experiments wherein known numbers of mutants were added to the plates to determine the effects on mutant expression of crowding by the non-mutant background (see Ryan, 1953). After 2·5 days, guanine-independent plate mutant colonies appear; i.e. mutants not present in the growth tubes that arise on the plates from the parental guanine-dependent cells; therefore, the counts were made at this time. Several colonies were isolated from the streptomycin and minimal plates; after numerous transfers it was demonstrated that these bacteria retained their mutant characteristics. The numbers of viable organisms were measured by dilution and seeding into Difco nutrient agar pour-plates. All determinations of the frequency of mutants were made with a minimum of five replicate cultures.

Only inhibitory compounds were found to be mutagenic; however, non-inhibitory concentrations of NHB (10 and 20 μg./ml.) were also mutagenic. No instance of specific mutagenesis was demonstrated; that is, no case of a mutagen affecting one marker and not the other. The streptomycin locus (loci) is affected much less by 6-nitrobenzimidazole than is the guanine locus (loci); nevertheless, this low degree of mutagenesis at the streptomycin locus is reproducible in repeated experiments. Although the frequency of spontaneous guanine-independent mutants is approximately a hundred-fold greater than the frequency of spontaneous streptomycin-resistant mutants, the induced factor of increase is greater at the streptomycin locus in one case, greater at the nutritional marker in others, and equal with two of the compounds. Similar studies on specific mutagenesis were made by Ryan (1952), who found no mutagenic activity for analogues of β-galactosides at the locus involving ability to synthesize β-galactosidase.

The fact that these mutagens actually increase the number of mutations to purine independence as determined by the papilla method was more readily demonstrated with NHB than with any other active compound (Table 2). This analogue also greatly increases the frequency of streptomycin-resistant mutants (Table 1) which made it suitable for relatively simple antimutagen-screening (washing bacteria was not necessary before plating for streptomycin-resistant mutants). Therefore, extensive studies on the mutagenesis of this compound were undertaken.

Table 2. *The relationship between the concentration of* 4-NO_2-6-OH *benzimidazole and the mutation rate in strain Wp⁻ from purine dependence to independence as determined by the papilla method*

Concentration of NHB* (µg./ml.)	Percentage of *c.* 500 colonies having number of papillae equal to: 0	1	2	3, 4	5–15	Mean number of papillae/ colony	Mean number of bacteria/ colony ($\times 10^6$)	Mutation rate bacterium/ generation ($\times 10^{-10}$)	Factor of increase over spontaneous rate
0	90	7·4	2·1	0·45	0·0	0·130	19·0	47·0	—
15	84	12	3·2	0·79	0·0	0·216	16·4	91·2	1·9
35	74	22	3·5	0·20	0·0	0·305	9·00	235	5·0
43	57	33	6·2	3·9	0·30	0·600	6·45	649	14
55	34	28	15	14	9·4	1·68	6·07	1910	41

* There were too many papillae/colony to measure at a concentration of 85 µg./ml.

Mutagenic activity of 4-nitro, 6-hydroxybenzimidazole

Table 2 shows the relationship between the concentration of NHB and the mutation rate of *Escherichia coli* Wp⁻ at the purine locus (loci) as determined by the papilla method. This method has been described by Ryan, Schwartz & Fried (1955). About 200 Wp⁻ bacteria were spread with a glass rod on the surface of 30 ml. minimal agar in Petri dishes containing 1 µg. guanine/ml., 0·5 % (w/v) glucose and the compounds under study in appropriate concentrations. The suboptimal concentration of guanine limits the size of the colony so that it contains only 10^7 bacteria. After 5 days, guanine-independent mutants visibly express their selective advantage over the guanine-dependent cells and form papillae which may be readily counted under low power with a binocular microscope. Papillae counts were made at 9 days. The total number of bacteria/colony was determined by suspending a colony having no papillae in 1 ml. of saline and counting the bacteria in a Petroff-Hausser bacteria counter. This total count was done for colonies of different sizes on control and experimental plates. To investigate the possibility that as many mutant bacteria occur on plates not containing the mutagen as on experimental plates but are expressed as papillae only in the presence of the analogue, several colonies from experimental and control plates containing no papillae were resuspended in saline and plated and found to contain no mutants.

Table 3 summarizes the mutagenic effect of NHB on *Escherichia coli* Wp⁻ under conditions which permit little or no cell division; in saline, minimal medium devoid of glucose, or long exposure in the stationary phase of growth. To determine induced mutagenesis in non-growing conditions, 5×10^9 bacteria were washed twice and suspended in 5 ml. of 0·85% (w/v) saline or media devoid of glucose and aerated in test tubes on a roller at 37°. After the organisms were exposed to the compound, they were washed twice and plated in minimal and nutrient agar for a determination of the frequency of guanine-independent mutants. A sample of washed bacteria containing 10^8 organisms was then inoculated into minimal medium supplemented with 65 μg. guanine/ml. wherein the bacteria underwent intermediate cultivation as indicated in Table 3. The bacteria were then washed twice and plated. The analogue had no mutagenic activity under non-growing conditions. There was no significant mutagenesis during long exposures to these conditions, even after intermediate cultivation in supplemented minimal medium which allows for the expression or recovery of mutants induced in the stationary phase. The compound, NHB, had a lethal effect in these non-dividing conditions. Induced mutagenesis may have occurred if an energy source had been made available to the bacteria; however, this was not tested. In a few experiments there was a great increase

Table 3. *The mutagenic effect of* 4-NO_2-6-OH *benzimidazole in* Escherichia coli *Wp⁻ under conditions which permit little or no cell division: in saline, minimal medium devoid of glucose and long exposure in the stationary phase of growth*

Conditions of exposure to mutagen		Concn. of NHB (μg./ml.)	Mean factor of increase over spontaneous purine independent mutant frequency	Mean factor of increase over shorter exposure
A.	18 hr. of logarithmic growth	40	5·0	.
B.	5 hr. of stationary phase exposure following 18 hr of logarithmic growth	40	4·0	.
B′.	Condition B+5 generations of intermediate cultivation	40	8·2	.
C.	28 hr. of stationary phase exposure following 18 hr. of logarithmic growth	40	4·7	1·2
C′.	Condition C+5 generations of intermediate cultivation	40	5·9	0·72
D.	14 hr. of exposure in 0·85% saline	40	0·81	.
D′.	Condition D+5 generations of intermediate cultivation	40	1·7	.
E.	40 hr. of exposure in 0·85% saline	40	1·6	2·0
E′.	Condition E+5 generations of intermediate cultivation	40	1·8	1·1
F.	48 hr. of exposure in media devoid of glucose	80	1·5	.
F′.	Condition F+11 generations of intermediate cultivation	80	0·30	.

in the spontaneous mutant-frequency in stationary conditions. This phenomenon could not be demonstrated in later experiments. Similar increases have been found in Streptomyces by Wainwright (1956) and in *E. coli* by Ryan (1955*b*).

Incorporation of compound NHB into DNA

NHB was not incorporated into the DNA of *Escherichia coli* Wp$^-$. In one experiment chromatographic analysis was performed on a population consisting of over 99·9 % mutants, the great majority of which were grown from mutants induced by the compound NHB. The bacteria that were analyzed for incorporation of the analogue were obtained in the following two ways:

(1) 10^6 bacteria were inoculated into each of four 4 l. flasks containing 3 l. minimal medium supplemented with 65 μg. guanine/ml. In addition, three flasks contained 50 μg. 4-nitro-6-hydroxybenzimidazole/ml. The cultures were aerated by bubbling sterile air into the flasks. By restricting the glucose to 0·1 % (w/v), the bacteria reached a concentration of 2×10^9 viable organisms/ml. The cells were maintained in the stationary phase of growth for a period of 24 hr., washed twice and their DNA subjected to paper chromatographic analysis.

(2) The same technique was used as in (1) with these modifications: the growth of the bacteria was limited to 2×10^6 organisms/ml. by the presence of only 2 μg. guanine/ml., so that induced mutants would have a selective advantage, overgrow and comprise the majority of the population. Because these organisms utilize glucose in the guanine-limited stationary phase, glucose was added to a concentration of 0·4 % to enable the mutants to overgrow the guanine-dependent population. The frequency of guanine-independent mutants was determined by differential plating in minimal and nutrient agar each day. On the fourth day, the bacterial population exhausted the glucose from the medium while the control culture growing in the absence of NHB reached this secondary stationary phase at 5 days. Each culture was maintained in the stationary phase for 24 hr. and the DNA was isolated and analysed. The isolation of DNA and the chromatographic analysis was performed by Drs M. Engelman and S. Graff (Francis Delafield Hospital, New York) by the method of Smith & Wyatt (1951). NHB was not detected in the perchloric acid-hydrolysed DNA.

Antimutagenesis

The effects of DNA and ribonucleic acid, an acid and alkaline hydrolysate of each, ribotides and ribosides (including xanthosine and inosine) singly and in combination, some deoxyribosides, purines and pyrimidines and some of their precursors (including aminoimidazolecarboxamide and orotic acid), purine and pyrimidine analogues, indole, vitamin B_{12}, riboflavin, folic acid, *p*-aminobenzoic acid and other vitamins were tested as antagonists, in various concentrations to growth inhibition and mutagenesis by compound NHB. To determine the antimutagenic activity of these compounds, 10^5 Wp$^-$ bacteria were inoculated into 6 ml. of media, supplemented with 6 μg. guanine/ml. containing the antimutagen under study, in test tubes. After 5 hr. (lag phase

+c. 2 generations) compound NHB was added aseptically to a concentration of 40 μg./ml. Periodic determinations of the turbidity were made in a colorimeter to study the annulment of growth inhibition by the test antimutagens. After 15 generations of growth under these conditions, the unwashed stationary-phase bacteria of each tube were plated in Petri dishes to which minimal 2% (w/v) agar with 100 μg. streptomycin/ml. + 65 μg. guanine/ml. was added. The number of streptomycin-resistant colonies and the number of viable bacteria was determined in the usual manner. It was determined that compound NHB had no additional effect when carried over with the undiluted, unwashed bacteria into the streptomycin-resistant mutant assay plates. None of the compounds or mixtures reversed the growth inhibition or mutagenesis of the analogue although several, including 5-aminouracil and a vitamin mixture, stimulated bacterial growth considerably.

Antimutagenesis by amino acids. Complex media which included yeast extract, Proteose-peptone, and Casamino acids were then tested as antimutagens. They were found to be active in depressing (20- to 80-fold) the frequency of mutants induced by NHB. Before examining the antimutagenesis of amino acids, studies were made to determine whether the activity of complex media was due *solely* to the great decrease in generation time that occurred in the presence of these agents. It was found that growing the bacteria at 25° instead of 37° increases both the generation time and the frequency of induced mutants; however, this temperature had no effect on the marked antimutagenesis of Casamino acids or yeast extract even though the generation time of bacteria growing in the presence of compound NHB + antimutagen is extended from 35 to 90 min. at the lower temperature. At 15° the antimutagenic effect of complex media is also unchanged in spite of the fact that the generation time of the bacteria growing in the presence of compound NHB (+antimutagen) is extended considerably. Although the effect of changes in temperature on growing cells is not clearly understood, these experiments indicated that something other than decreased generation time was involved in the high antimutagenic activity of complex media. Experiments utilizing various concentrations of Casamino acids and the mutagen also showed the antimutagenic activity to be relatively independent of a lower generation time; for example, while there is only a slight change in the growth rate brought about by a 1000-fold decrease in concentration of casein hydrolysate (from 20 mg. /ml. to 20 μg./ml.) there is a marked decrease in antimutagenic activity. These findings, as well as the finding that 5-aminouracil and a vitamin mixture have no effect on mutagenesis even though they decrease the generation time considerably, led to a series of experiments which were designed to resolve the activity of hydrolysed casein into the activity of one or more of its constituent amino acids.

A mixture of 20 amino acids was as effective as casein hydrolysate in decreasing the mutagenic activity of compound NHB. The frequency of induced strepromycin-resistant mutants was decreased 20- to 30-fold. No single amino acid was found to be effective. Many concentrations and ratios were tested. Serine was the only indispensable amino acid. Only high concentrations of the amino acids, of the order of 0·1–1·0 mg./ml., were effective. Combinations of amino acids that result in widely different generation times are antimutagenic to the same degree and combinations that result in the same high growth rate have diverse antimutagenic properties. Glutamic acid, for example, was as effective as all the amino acids combined in increasing the rate of growth; however, it had very little effect on the mutagenesis of NHB. None of the amino acid combinations nor complex media annulled the inhibitory activity of the analogue; furthermore, some combinations were inhibitory to growth.

Table 4. *The antimutagenic activity of amino acids on the mutagenesis of* 4-NO$_2$-6-OH *benzimidazole in* Escherichia coli *Wp$^-$ as measured by the papilla method*

Amino acids and concentration in μM		Concentration of NHB (μM)	Purine$^-$ to purine$^+$ mean mutation rate/bacterium/gen. ($\times 10^{-10}$)	Mean factor of increase over spontaneous rate	Mean factor of decrease of induced mutation rate
0	—	0	108	.	.
Glutamic acid Serine Methionine	6800 8550 3850	0	89·2	0·81	.
Glutamic acid Serine Alanine Tryptophan	13600 17100 10100 2820	0	139	1·3	.
0	—	224 (40 μg./ml.)	1310	12	.
Glutamic acid Serine Methionine	6800 8550 3850	224	92·3	0·86	14
Glutamic acid Serine Alanine Tryptophan	13600 17100 10100 2820	224	493	4·6	2·7

Table 4 summarizes the effect of two amino acid combinations on the NHB-induced mutation rate. The amino acid combinations did not decrease the spontaneous mutation rate as measured directly by the papilla method nor did they, as separate experiments show, affect the frequency of spontaneous guanine-independent and streptomycin-resistant mutants in liquid cultures.

The antimutagenic activity of the two amino acid combinations in the presence of the mutagen was then determined. At high concentrations of NHB (60–80 μg./ml.) there was very little reversal by the amino acids. At the lower concentrations of the mutagen the results obtained are consistent with results that would be expected in a competitive system. However, the non-linearity of the antimutagenic effect of different concentrations of the amino acids obtained at one mutagen concentration, and the non-linearity of the mutagenic effect of increasing mutagen concentrations at one amino acid concentration, indicated that this is an extremely complex system, not easily subjected to competitive reversal analysis. The degree of reversal of induced mutagenesis was found to be independent of the molar ratios of mutagen to antimutagen at most concentrations. Although 40 μg./ml. of the mutagen increased the nutritional mutant frequency 44-fold, and elevated the streptomycin-resistant mutant frequency 290-fold, the factor of depression of the induced mutant frequency by the amino acid combinations was almost identical for each locus. This is true for almost all concentrations.

Pre- and post-treatment experiments with amino acids. The pre- and post-treatment effects of amino acids on the mutagenesis by the analogue are indicated in Table 5. The concentration of NHB used in all pre- and post-treatment experiments was 35 μg./ml.; the amino acid concentrations were L-glutamic acid, 1000 μg./ml., DL-serine, 900 μg./ml., and DL-methionine, 575 μg./ml. Minimal media was supplemented with 65 μg. guanine/ml.

Table 5. *The effect of amino acids* on the frequency of chemically-induced mutants before and after treatment with* 4-NO$_2$-6-OH *benzimidazole in* Escherichia coli Wp^-

I. *Pre-treatment experiment*

E. coli Wp$^-$ was grown in supplemented minimal medium with and without amino acids (pre-treatment) and then grown in media described below (subsequent treatment):

	Mean mutant frequency × 10^{-9}/bacterium purine$^-$ to purine$^+$ Pre-treatment	
Subsequent treatment	Minimal medium	MM. + amino acids
Minimal medium (MM)	2·3	3·0
MM + amino acids	4·0	3·9
MM + 35 μg. NHB/ml.	200	190
MM + amino acids + 35 μg. NHB/ml.	67	67
Cells washed; then MM	2·5	2·5
Cells washed; then MM + 35 μg. NHB/ml.	330	230

II. *Post-treatment experiment*

E. coli Wp$^-$ was grown in MM with and without NHB (treatment) and then grown in MM with and without amino acids (post-treatment):

	Post-treatment	
	Minimal medium	MM + amino acids
Treatment (5 generations)	(*a*) in agar (unsupplemented)	
Minimal medium	7·5	7·0
MM + 35 μg. NHB/ml.	680	660
	(*b*) in liquid (5 gen.)	
Minimal medium	10	9·3
MM + 35 μg. NHB/ml.	180	260

* Amino acids = glutamic acid 1000 μg./ml.; serine, 900 μg./ml.; methionine, 575 μg./ml.

In pre-treatment experiments 5×10^4 bacteria were introduced into 5 ml. of minimal medium and of minimal medium plus amino acids and grown for 15 generations. When the bacteria reached a population size of 10^8 bacteria/ml. (3 generations before saturation) they were subsequently treated as indicated in Table 5. The inoculum introduced into tubes containing 5 ml. of treatment media was 5×10^6 bacteria. When these bacteria reached the stationary phase of growth, mutant frequencies were determined. In post-treatment experiments an inoculum of 10^5 guanine-dependent cells was introduced into 5 ml. of treatment media (see Table 5) and grown for 10 generations under standard conditions. Three generations before saturation some of the logarithmic-phase bacteria were plated in minimal agar and minimal agar containing amino acids after the cells were washed. Approximately 8×10^7 bacteria of this washed suspension were inoculated into 8 ml. of supplemented minimal medium and supplemented minimal containing amino acids, wherein the bacteria grew for 5 generations to stationary phase. The stationary-phase cells were then washed once and plated to determine the frequency of guanine-independent mutants. Growing the bacteria in amino acids had no effect on the subsequent mutagenesis of the analogue or on the subsequent antimutagenesis of amino acids, nor did the presence of amino acids in liquid or agar after treatment with the mutagen have any effect on the mutagenic activity of the analogue. Under the conditions tested, the antimutagenic effect of the amino acid combinations occurred only when they were present simultaneously with the mutagen.

If the effect of pre-treatment is solely on the very early generations in the treatment media containing the analogue, it would not be easily detected in the present experiments because the majority of the mutants are induced in the terminal generations of growth. In view of the findings of Witkin (1956), Ryan (1954, 1955*a*) and Ryan, Fried & Schwartz, (1954) the number of generations in post-treatment media may not be as critical as the rate of growth in the first generation after treatment. If post-treatment effects can only be expressed when they are applied before a bacterial division occurs as is the case with photo-reactivation and some chemical post-treatment effects on ultra-violet light, their detection in the present system would be improbable since the reversal would affect only one generation (the last) of induced mutants. However, it should be noted that in treatment medium as many divisions (and consequently as many mutations) occur in the last division as in the sum of all the preceding generations.

The amino acids do not appear to act by forming a complex with the analogue without the intervention of a biological reaction as in the instance of nucleic acid-binding acriflavine, and thus interfering with the latter's mutagenic activity in *Escherichia coli* (Witkin, 1950). The antimutagenic effect of amino acids is no greater if the cells are inoculated into tubes in which the analogue and the amino acids have been incubated together at 37° for 25 days or if the cells are inoculated into tubes containing the analogue and the amino acids are added after 5 hr. of growth. Further evidence against simple chemical binding is the fact that the antimutagenic effect of amino acids is strain specific and does not occur in B/r, t, where in some experiments the amino acids actually enhance mutagenesis.

Antimutagenesis by analogues of compound NHB. The antimutagenic effect of 4-hydroxy-6-nitrobenzimidazole and 4-hydroxy-6-nitrobenzotriazole on the mutagenesis of NHB in liquid cultures is summarized in Table 6. Neither of these compounds is mutagenic and both inhibit the growth of the bacteria. The spontaneous mutant-frequency is not affected by 4-hydroxy-6-nitrobenzotriazole, yet the compound depresses the induced mutant frequency significantly ($P=0{\cdot}001$ for each marker both before and after intermediate cultivation for 6 generations) in minimal medium supplemented with guanine. Other analogues of NHB in addition to 4-hydroxy-6-nitrobenzimidazole, which had no effect on the NHB-induced mutant frequency (not indicated in tabular form) are: 2:4-dihydroxy-6-nitrobenzimidazole, 2-methyl-4-hydroxy-6-nitrobenzimidazole, 4-nitro-6-methoxybenzimidazole, 2-ribotyl-4-nitro-6-methoxybenzimidazole, 4-nitro-6-ethoxybenzimidazole and 5-nitro-7-methoxyquinoxaline.

Table 6. *The antimutagenic effect of two analogues of* 4-NO_2-6-OH *benzimidazole in* Escherichia coli *Wp⁻*; 4-OH-6-NO_2 *benzimidazole and* 4-OH-6-NO_2 *benzotriazole*

Test antagonist*		None			4-OH-6-NO_2 benzimidazole 559 μM			4-OH-6NO_2 benzotriazole 695 μM		
Marker	Time of plating	Spont. m.f.† (*a*)	196 μM (35 μg./ml.) NHB induced m.f.† (*b*)	Mean factor of increase (*b*/*a*)	NHB m.f.†	196 μM NHB m.f.† (*c*)	Mean factor of decrease (*b*/*c*)	NHB m.f.†	196 μM NHB m.f.† (*d*)	Mean factor of decrease (*b*/*d*)
Purine p^- to p^+	Zero	9·3	953	103	46	442	2·2	25	173	5·5
	After intermediate cultivation	13	4190	320	14	3430	1·2	7·0	975	4·3
Streptomycin S^s to S^r	Zero	0·25	137	550	1·6	28·4	4·8	0·25	14·8	9·2
	After intermediate cultivation	1·0	205	205	0·80	254	0·80	4·0	32·4	6·3

* Antagonist was added to growing bacteria in liquid cultures 3 hr. before addition of NHB.
† m.f. = mean mutant frequency × 10^{-9}/bacterium.

Mutagenesis and antimutagenesis by some compounds related to nucleic acid metabolism

The mutagenic activity of several inhibitors and inhibitor antagonists on 5 loci in 5 mutants of *Escherichia coli* are summarized in Table 7. The bacteria grew in the presence of these compounds for 15 generations; the exposure to the chemicals was continued for 24 hr. in stationary phase conditions; this was followed by a period of intermediate cultivation for 5 generations, washing in saline, and plating for the determination of the mutant frequency. NHB, 5-nitroquinoxaline, and 5-aminouracil are general in their effect. *E. coli* 15h⁻ appears to be the most mutagen-stable, while B/r, t is the most mutagén-sensitive. There is no correlation between the spontaneous mutant frequency and the factor of increase of a single compound from one strain to another; for example, NHB has no effect on *E. coli* 15m⁻, although the spontaneous mutant frequency is approximately 10^{-5} mutants/bacterium. There are no major differences in response of these strains to the inhibitory action of these compounds. Bacterial growth is not inhibited by 5-aminouracil but it is a potent mutagen.

Table 7. *The mutagenic activity of several inhibitors and antagonists to inhibition in 5 mutants of* Escherichia coli*

Strain:	...	...	Wp$^-$	15h$^-$	15h$^+$m$^-$	B/r,m$^-$	B/r
Marker tested:	...	...	S^s to S^r	h$^-$ to h$^+$	m$^-$ to m$^+$	m$^-$ to m$^+$	t$^-$ to t$^+$
Spontaneous mutant frequency $\times 10^{-10}$/bact.:	...	...	3	8400	90,000	10	20
Compound	(μg./ml.)	Effect on growth†	Induced factor of increase over spontaneous mutant-frequency‡				
2,6-Diaminopurine	250	++	4	0·76	1·6	**79**	3
	325	+++	.	.	2·7	.	.
6-Chloropurine	400	−	0·8	0·50	1·3	2	1
	700	−	.	.	2·2	.	.
2-Thioadenine	250	−	3	0·89	1·2	1	1
6-Mercaptopurine	100	+++	.	.	**7·8**	.	1
	150	++++	.	0·5	.	.	.
Dithio-6-oxypurine	1000	−	.	.	1·7	.	2
4-Amino, 5-imidazole-carboxamide	500	−	4	0·80	1·1	1	.
8-Azaguanine	500	+	1	.	2·1	1	1
Caffeine	250	−	.	.	1·6	.	1
	500	−	.	.	.	.	**6**
Theophylline	500	−	.	.	2·1	1	1
Benzimidazole	300	+++	1	1·1	.	.	**14**
	800	++++	.	.	0·50	1	**31**
4-NO$_2$-6-OH—	40	+	**370**	0·60	.	.	**26**
Benzimidazole	80	++	.	**4·7**	1·6	**10**	**37**
5-Aminouracil	300	−	**7**	.	.	.	**6**
	500	−	.	1·6	**7·0**	.	.
	700	−	.	.	**14**	2	**48**
2-Amino-4-methyl-pyrimidine	1000	−	.	.	1·5	.	1
Barbituric acid	1000	−	.	.	1·1	.	.
Uric acid	1000	−	.	.	1·1	.	.
Thiouracil	1000	+	.	.	1·4	.	1
Aminopterin	100	++++	.	0·43	0·82	1	1
Ca leucovorin	1000	−	1	.	1·3	.	.
5-Nitroquinoxaline	15	++	.	.	**4·6**	.	.
	20	++	**7**	0·41	**41**	.	.
	25	+++	.	.	**59**	**47**	**19**
Sulphanilamide	2000	++++	.	0·45	1·5	.	.
Sulphathiazole	500	++++	1	.	.	.	.
2-Chloro-4-aminobenzoic acid	500	+	.	.	.	.	2
p-Aminobenzoic acid	1000	−	1	0·60	0·91	.	1

Figures in black show greater than 4-fold mutagenic activity.

* Abbreviations: p, purine; S, streptomycin; h, histidine; m, methionine, t, tryptophan; $^-$, dependence $^+$, independence; s, sensitivity; r, resistance.

† See Table 1 for definition of degree of inhibition.

‡ Mutant-frequencies were determined after: (*a*) growth of bacteria in liquid cultures containing test mutagen; (*b*) continued long exposure of bacteria in stationary phase of growth to test mutagen; (*c*) intermediat cultivation in appropriately supplemented minimal medium.

The effects of several compounds on the mutagenesis on NHB, benzimidazole, 5-nitroquinoxaline, 5-aminouracil, and 6-mercaptopurine are summarized in Table 8. For example, in the presence of 300 μg. 5-aminouracil/ml. the induced frequency of purine-independent mutants in *E. coli* Wp$^-$ is 173×10^{-9}/bacterium. This is a 17-fold increase over the spontaneous mutant frequency of $10 \cdot 2 \times 10^{-9}$/bacterium. In the presence of an amino acid mixture and 5-aminouracil the mean mutant-frequency is 127×10^{-7}/bacterium—only 1·4-fold lower than the induced mutant frequency in the absence of amino acids. The antimutagenic activity of the combination of glutamic acid, serine, and methionine is strain specific; they do not depress the mutagenic activity of NHB and 5-nitroquinoxaline in any strain other than Wp^-. Furthermore, the amino acids have no effect on the mutagenic action of 5-aminouracil in *Escherichia coli* Wp^-. In all cases, the amino acids stimulated growth considerably. The mutagenesis of 5-aminouracil cannot be reversed by any of the compounds or mixtures employed in these studies. Purines and purine ribosides reverse the growth inhibition and the mutagenic activity of 6-mercaptopurine; furthermore, the depression in the frequency of mutants is retained through 5 generations of intermediate cultivation in liquid medium. Folic acid does not affect the inhibition or mutagenesis of 6-mercaptopurine. 6-Mercaptopurine was found to be extremely inhibitory in the presence of low concentrations of amino acids. Although the inhibitory activity of benzimidazole is reversed by guanosine and adenosine in *E. coli* B/r, t there is no reversal of the mutagenesis of this compound. None of the test antagonists have any effect on the spontaneous mutant-frequency. This is also true for all the compounds tested as antagonists to the mutagenesis of NHB.

DISCUSSION

The analogues active as growth inhibitors in *Escherichia coli* are, in general, the same as those which were found to be inhibitory to developing *Rana pipiens* embryos by Liedke, Engelman & Graff (1954, 1955). An exception is NHB which does not affect the embryos. Loveless, Spoerl & Weismann (1954) have shown that 4-hydroxy-6-nitrobenzotriazole specifically inhibits cell division in yeast and *E. coli*; the proposed mechanism of inhibition is through the formation of a carbonium ion. This may be true for other related compounds employed in the present studies. Although Woolley (1944) has shown that benzimidazole inhibition can be reversed by adenine or guanine, there are several other investigations in which nucleic acid constituents failed to reverse benzimidazole inhibition (Kushner & Pascal, 1955; Pollock, 1947; Roblin *et al.* 1945). Novick & Szilard (1952), using high concentrations of ribosides, could not decrease by more than one-third the mutagenesis by 150 μg. benzimidazole/ml.

Because induced mutagenesis was found to occur only under growing conditions, a mechanism involving the selection of pre-existing mutants by the analogue seemed likely; however, the papilla method of determining the mutation rate demonstrates that the action of NHB results in a greater number of mutational *events* rather than simply a greater number of mutants. Ryan *et al.* (1955) have shown that NHB increases the mutation rate at the histidine locus fourfold as measured by the papilla method in *Escherichia coli* 15, h^-. The fact that there is an increase in streptomycin-resistant mutants without any detectable killing or growth inhibition in liquid cultures suggests

Table 8. *The effect of various compounds on the mutagenesis by* 4-NO_2-6-OH *benzimidazole, benzimidazole, 5-nitroquinoxaline, 5-aminouracil, and 6-mercaptopurine in 3 strains of* Escherichia coli

(1) = Strain (2) = Marker (3) = Mean spontaneous mutant frequency $\times 10^{-9}$/bact. (*a*)	Mutagen	Concn. (μg./ml.)	Mean induced mutant frequency ($\times 10^{-9}$) (*b*)	Mean factor of increase (*b*/*a*)	Test antimutagen*	Concn. (μg./ml.)	Mean mutant frequency $\times 10^{-9}$/bact. mutagen + antagonist (*c*)	Mean factor of decrease (*b*/*c*)
(1) Wp^- (2) p^- to p^+ (3) 10·2, 29·0	5-Aminouracil	300	173	17	aa†	.	127·0	1·4
	5-Nitroquinoxaline	20	495	17	aa	.	68·0	7·3
(1) $15h^-m^-$ (2) m^- to m^+ (3) 122	5-Nitroquinoxaline	15	7240	59	aa	.	4500	1·6
					Folic acid	100	5060	1·4
	6-Mercaptopurine	90	2410	20	Adenosine	250	61·0	40
					Guanosine	250	231	10
					Adenine	250	111	22
						83	238	10
					Guanine	250	41·0	59
						83	127	19
					Folic acid	100	2660	0·90
(3) 59·0	5-Aminouracil	750	810	14	aa	.	2580	0·31
					Guanosine Adenosine Thymidine Uridine Cytidine	250	780	1·0
					Thymine	250	617	1·3
					Uracil	250	498	1·6

(1) B/r, t (2) t^- to t^+ (3) 4·95–7·05	5-Aminouracil	750	558	110	aa	.	697	0·80
					Guanosine Adenosine	500	354	1·6
					Thymidine	800	428	1·3
					Uridine	800	350	1·6
					Cytidine	800	437	1·3
		300	44·0	6·3	Thymidine	1000	118	0·37
					Uridine	1000	94·1	0·47
					Cytidine	1000	94·4	0·47
	Benzimidazole	200	96·1	14	aa	.	65·2	1·5
					Vit. B_{12}	16	184	0·52
					Guanosine Adenosine	500	126	0·76
	$4\text{-}NO_2\text{-}6\text{-}OH$ Benzimidazole	75	261	37	aa	.	344	0·76
		75	402	80	aa	.	620	0·65
		40	215	31	aa	.	1170	0·18
		40	129	26	aa	.	119	1·1
		15	70·4	14	aa	.	33·1	2·1
		75	261	37	Vit. B_{12}	16	242	1·1
					Guanosine Adenosine	500	549	0·48

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* Test antimutagen was added to growing cells in liquid cultures 3 hr. before addition of mutagen. When the bacteria subsequently reached the stationary phase of growth they were washed and the frequency of prototrophs was determined by plating in minimal and nutrient agar.

† aa: L-glutamic acid, 1000 μg./ml.; DL-serine, 900 μg./ml.; DL-methionine, 575 μg./ml.

that the analogue is not acting by decreasing the proportion of parental cells. Even at concentrations of the compound which kill, there is an *absolute* increase in the number of mutants. In addition, mutant frequencies of six different markers have been shown to be affected by NHB. Several different mechanisms would have to be postulated for the selective action of NHB against amino acid-requiring strains, purine-requiring strains, streptomycin-sensitive strains and selection for streptomycin-resistant organisms; on the other hand, the single mechanism of increased genetic instability could account for these results. Moreover, in the presence of NHB, auxotrophs, prototrophs, streptoymcin-resistant and -sensitive cells grow, independently, at the same rate. Thus the action of NHB is thought to involve a mechanism whereby the rate of mutation is increased.

The action of NHB is very different from that of the mustards and most other mutagens. Because of the elimination of many selection problems and lack of clonal variation in conditions of exposure in stationary phase, almost all mutagen-screening studies have been made with non-growing bacteria. If the same techniques were employed in the present studies, the mutagenic activity of the compounds would not have been detected. This may imply that these compounds, as well as caffeine and theophylline, do not act via a free radical mechanism or that they have a unique mode of action since formaldehyde, peroxides, the mustards, acriflavin and the Mn ion act on stationary populations. These findings are consistent with those of Lee (1953), who has shown that caffeine and theophylline are not mutagenic at very long generation times.

Analogues of nucleic acid constituents possessing conjugate bond stability, and which do not inactivate Transforming Principle (Zamenhof, Leidy, Hahn & Alexander, 1956), more likely act by interfering with DNA synthesis. Because benzimidazole is known to inhibit the activity and synthesis of several enzymes, it is possible that benzimidazole derivatives also inhibit enzymes concerned with nucleic acid synthesis. This seems likely in view of the studies of Koch & Lamont (1956) who have shown that caffeine, theophylline and theobromine (as well as some natural bases) inhibit some enzymes involved in nucleic acid metabolism.

If there is any incorporation into DNA of compound NHB at all, it is far less than the incorporation found for 5-bromouracil (Zamenhof & Griboff, 1954) and 6-methyladenine (Dunn & Smith, 1955) in *Escherichia coli* and for 8-azaguanine in the RNA of *Bacillus cereus* (Mathews & Smith, 1956). The studies of Koch (1956) have shown that radioactive caffeine and theophylline are not incorporated into bacterial DNA. They failed to detect (as did the present studies) incorporation of these analogues into analogue-induced mutants.

Although their ability to decrease the generation time may not be solely responsible for the antimutagenesis of amino acids, the effect on the growth rate may contribute to decreasing the efficiency of the action of the mutagen. The antimutagenic activity of amino acids may be due to the role they play in the synthesis of purines and pyrimidines whereby they could supply the bacteria with 'correct' nucleic acid constituents. The simplest amino acid combination (serine, glutamic acid and methionine) found empirically to be the most effective antimutagen, contains amino acids which have been shown to be actively concerned with the synthesis of purines and pyrimidines (see Reichard, 1955, and Schlenk, 1955). For example, glutamic acid may contribute to the amino group on pyrimidines and to N_1 of the ring. Serine takes part in the formation of C_2, C_4, C_8 and N_7 of purines and the methyl group of thymine. Methionine can act as a methyl donor in the conversion of aminoimidazolecarboxamide to purines. NHB may interfere with vitamin B_{12} activity (this vitamin contains 5–6-dimethylbenzimidazole and is involved in thymine synthesis). Although vitamin B_{12} had no effect on the activity of NHB, methionine, a methyl donor which can substitute for vitamin B_{12} in many reactions, may be involved in this system. Vitamin B_{12} can reverse substituted benzimidazole inhibition in a B_{12}- or methionine-requiring mutant of *Escherichia coli* strain W (Scott, Rogers, Rose & Chu, 1957).

The mechanism of the reversal of the mutagenic activity of 5-nitroquinoxaline by amino acids (see Table 7) is subject to similar speculation. Folic acid and the citrovorum factor are involved in single carbon unit metabolism and are concerned with the synthesis of formate (a purine and pyrimidine precursor) from glycine and serine; therefore, the addition of exogenous serine may circumvent the inhibited reaction. It is also possible that glutamic acid, together with *p*-aminobenzoic acid (*p*-AB), acts as a trap for the pterin analogue, since, normally, *p*-AB, glutamic acid and pterin form folic acid.

Adding 'ready made' amino acids may increase protein synthesis; this may then lead to a number of changes which could affect the mutation process, such as an increase in the synthesis of enzymes involved in DNA duplication that may be blocked due to the possible ability of NHB to interfere with RNA synthesis which, in turn, appears to be necessary for protein synthesis. If NHB were interfering with the *action* of DNA-synthesizing enzymes as caffeine does in Koch's studies, then increased enzyme synthesis could antagonize this inhibition. Witkin (1956) has found that the mutant frequency of ultraviolet-induced tryptophan mutants is a function of the concentration of amino acids in the mutant-assay plates before the first bacterial division occurs. Witkin considers one possible explanation to be that increased protein synthesis leads to genetic repair of mutant cells that would normally die in absence of enrichment. If increased protein synthesis results in reversal of genetic alteration (reversal of mutation) as well as reversal of genetic killing of potential mutant cells, the results obtained in the present work may be reconciled with Witkin's findings, in spite of the fact that a decrease rather than an increase in the mutant frequency is obtained. The repair in Witkin's instance, may, however, be repair of the physiological damage to bacteria that have received ultraviolet irradiation; these are the organisms from which the mutants arise. It should be noted that the amino acids did not decrease the frequency of NHB-induced mutants in *Escherichia coli* B/r,t, the same strain in which Witkin found an increase in ultraviolet-induced prototrophs with amino acid post-treatment; furthermore, in the present system there is little killing by the mutagenic agent.

The absence of an effect of the amino acids on the spontaneous mutant frequency or mutation rate indicates that a selection mechanism is not involved in the antimutagenesis of amino acids, and suggests that spontaneous mutations may not be caused by benzimidazole-like compounds in the cell. The amino acids actually decrease the number of mutational events induced by NHB as has been demonstrated by the papilla method. These properties of the amino acid combination, in addition to their equal effect on the growth rate of mutant and parental cells, argue against a selection mechanism. The results obtained in pre- and post-treatment experiments also support an antimutagenesis mechanism; for if amino acids selected against induced or spontaneous mutants, pre- and post-treatment with amino acids would have had an effect on the frequency of mutants. Furthermore, a single concentration of amino acids does not result in a decrease of a constant number of induced mutants. The antimutagenesis of amino acids depends, to a limited degree, on the concentration of NHB (fewer mutants are annulled at lower concentrations of NHB).

None of the non-mutagenic analogues are as effective as 4-hydroxy, 6-nitrobenzotriazole in reducing the 4-nitro-6-hydroxybenzimidazole induced-mutant frequency. The antimutagenesis does not appear to be the result of inhibition alone since several other analogues which have similar inhibitory properties have no antimutagenic effect. Although studies on competitive reversal of induced mutagenesis were not pursued, an attractive hypothesis for the action of 4-hydroxy-6-nitrobenzotriazole is that it replaces NHB at certain sites in the cell.

Novick & Szilard (1951) and Novick (1955) found none of the pyrimidine analogues they tested to be mutagenic in *Escherichia coli* B/1, t at the T5- and T6-resistant loci; nevertheless, 5-aminouracil has been shown in the present studies to be an effective mutagen at several loci including the tryptophan locus of B/r, t. This is extremely interesting in view of Dunn & Smith's (1955) finding that a new purine, 6-methyladenine, replaces thymine in the presence of 5-aminouracil in *E. coli*. The mutagenesis of 5-aminouracil in these investigations suggests that the inability of pyrimidine analogues to act as mutagens in Novick & Szilard's studies was not due to the impermeability of the cell to the compounds as has been suggested by Marshak (Novick & Szilard, 1951). On the other hand, Novick & Szilard found that theophylline affects the T5 and T6 locus of B/r, t^-, whereas theophylline has no effect on the tryptophan locus in the present studies. This demonstrates the strain and locus specificity of mutagenic reactions. Consistent with the present findings are the results of Duncan & Woods (1953) who have shown that the effect of 5-aminouracil on chromosome breakage could not be reversed by thymine.

Although Novick & Szilard found no mutagenic activity for the components of the ribosides that were active antimutagens for caffeine and theophylline, in these studies the bases adenine and guanine, which annulled the growth inhibition of 6-mercaptopurine in these and other investigations (Elion, Hitchings & Vanderwerf, 1951), also annulled the mutagenic activity of the antimetabolite. This annulment by purine and pyrimidine bases indicates that the antimutagenic activity of ribosides is not due to the action of their ribose component as a trap for mutagenic purine and pyrimidine bases as Kalckar (1954) has suggested. The mechanism of reversal may be more simple and direct than that proposed for the antimutagenic activity of amino acids.

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Cultural, Cytological and Ecological Observations on the Amoeba Stage of *Naegleria gruberi*

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SUMMARY: A flagellate amoeba, isolated from Ohio River water and identified as *Naegleria gruberi* (Schardinger), has been maintained in permanent cultures on a buffered sucrose nitrate agar with its original bacterial associate, identified as *Proteus mirabilis*. On Czapek agar plates, the growth curve of the amoeba at 25–27° was found to consist of a lag phase of about 4 hr., a log growth phase of about 60 hr., and a growth-encystment phase of 2–3 weeks. At 37° the lag phase was shortened by half and the log growth phase by about a third. The generation time was 4½ hr. at 25–27° and 2½ hr. at 37°. Continuous cultivation at 37°, however, resulted in occurrence of abnormal forms. Very poor growth of amoeba on nutrient and tryptose agars was attributed to their high organic nitrogen content, poor growth in liquid media to the difficulty of amoebae to engulf bacteria. Attempts to grow the amoeba on heat-killed bacteria were failures. Redox potential and pH determinations revealed that the mixed fluid cultures were aerobic and the amoebae perished at pH values more acid than 5·6. Replacement of *Proteus mirabilis* by other bacteria by Oehler's technique was unsuccessful because of swarming but was successful with a *Flavobacterium* sp., which produced a yellow pigment whose antibiotic effect prevented swarming. Permanent cultures of the amoeba with the new bacterial associate were maintained after the amoeba overcame the antibiotic effect of the pigment. Replacement of the *Flavobacterium* sp. in the mixed culture by new species of bacteria was easily accomplished, and permanent cultures were maintained with *Aerobacter aerogenes, Escherichia coli*, 8 species of *Salmonella* and 2 of *Shigella*. The amoebae encysted earlier on all these species than on *Proteus mirabilis*.

Cytological observations include: (*a*) appearance of the chromatin material as short rods; (*b*) no evidence of thread-like structures radiating from the karyosome or chromatin patches in resting nucleus, nor the centrioles in mitosis; (*c*) karyosome origin of both the 'interzonal body' and the polar caps.

The cultivation of free-living amoebae has generally been done in broths in the presence of a heterogeneous group of bacteria normally found in water or soil. Growth in such cultures is usually very poor, due to overgrowth of inedible and/or toxic substance-producing bacteria (Singh, 1952). The unsatisfactory results obtained by these workers are reflected by the remark of Dobell (1914) that luck played a great part in determining their success. Credit must be given to Singh (1941, 1945, 1946, 1948, 1950) for developing the method of growing amoebae on non-nutrient agar supplemented with edible living bacteria. With this method, however, growth is limited by the supply of bacteria, and two types of cultures are needed for the maintenance of one.

Although the cytology of *Naegleria gruberi* (Schardinger) has been described in detail and many controversial claims have been clarified (Wilson, 1916; Zulueta, 1917; Kühn, 1920; Ivanić, 1936; Rafalko, 1947; Singh, 1952), gaps exist in our knowledge of this subject, especially of the flagellate form. Much remains to be studied of the growth pattern, cultural characteristics and ecological matters relating to both the amoeba and the flagellate form. With a strain of *N. gruberi* isolated from the Ohio River two studies were made to fill these gaps. The present report concerns observations pertaining to the amoeba form of this organism.

METHODS

Media. Czapek and other standard media were made from Difco Bacto dehydrated products. A buffered sucrose, lactose, maltose, or glucose nitrate agar was compounded with the following ingredients (%, w/v): 1, carbohydrate; 0·2, $NaNO_3$; 0·9, $NaHPO_4.7H_2O$; 0·04, KH_2PO_4; 0·01, $MgSO_4.7H_2O$; 0·001, $FeSO_4.7H_2O$; 1·5, agar. A buffered sucrose tryptose agar was prepared to contain (%, w/v): 1, sucrose; 0·2, tryptose; 0·6, $Na_2HPO_4.7H_2O$; 0·04, KH_2PO_4; 1·5, agar. The sucrose was omitted in preparing the buffered tryptose agar. For growing mixed cultures of the amoeba and a *Salmonella* or *Shigella* sp., the buffered sucrose trypose agar was supplemented with 0·1% (w/v) glucose. Agar was omitted when these media were prepared in liquid form. The pH value of the agar medium thus prepared was 7·4–7·5 and that of the liquid medium, 7·4–7·6.

All media were autoclaved at 15 lb./sq.in. for 15 min. Agar plates were poured to a maximum depth that permitted direct microscopic examination through the bottom of the dish. Agar slopes were made in screw-capped 25 × 150 mm. tubes. Liquid media were dispensed in tubes and in Petri dishes and, for studying pH value and redox potential changes, in vessels (55 mm. tall and 45 mm. diameter) stoppered with 3-hole rubber stoppers and capped with aluminium foil.

When practical, a potentiometer was used for pH value and potential measurements (Chang, 1946); otherwise, the pH changes were roughly indicated by bromothymol blue and methyl red incorporated separately into the media.

Bacteria. Besides the original bacterial associate (*Proteus mirabilis*), *Flavobacterium* sp., *Aerobacter aerogenes*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas pyocyanea*, *Sarcina lutea*, *Salmonella typhosa*, *S. paratyphi*, *S. schottmuelleri* (paratyphi B), *S. enteritidis*, *S. cholerae-suis*, *S. typhimurium*, *S. meleagridis*, *S. pullorum*, *Shigella dysenteriae* II (*ambigua*), and *Sh. sonnei* were also studied for their capacity to support amoeba growth. These species of *Salmonella* and *Shigella* were obtained from the Communicable Disease Center, Chamblee, Georgia, U.S.A., through Mr B. A. Kenner of the Center. All other species were isolated from Ohio River water.

Flagellate amoeba. The flagellate amoeba was isolated from Ohio River water on Czapek agar containing 100 units penicillin and 200 μg. streptomycin/ml. medium. The amoeba was discovered in a mucoid colony of a motile bacillus. After several transfers on the same medium, co-existence of an amoeba and a single species of Gram-negative bacillus became apparent in the mixed culture. When the antibiotics were omitted from the agar, a confluent mucoid growth covered almost the entire surface, due to bacterial swarming. Reliance on its bacterial associate for food was evidenced by confinement of the amoeba within the area of bacterial growth and by its feeding freely on the bacteria.

The morphology and cytology of this amoeba are, for the most part, identical with those described by Rafalko (1947) and Singh (1952) for *Naegleria gruberi*. When its trophozoites were found by accident to undergo flagellate transformation when suspended in water, it was identified as *N. gruberi*. The bacterial associate was isolated in pure cultures and was identified by biochemical reactions and infrared spectrophotometric characteristics (Riddle *et al.* 1956) as *Proteus mirabilis*. Transfers of pure mixed cultures on Czapek agar plates to slopes of the same medium, however, always resulted in rapid death of the amoebae while the *P. mirabilis* maintained rich growth. The death of the amoebae was found to be due to accumulated acids in the mucoid growth at the bottom of the slopes. When buffered sucrose nitrate agar replaced the Czapek agar, rich growth of amoebae was obtained in all slope subcultures.

Replacement of bacterial associate. Because of the relatively poor growth with Singh's method of cultivation, the agar-block technique (cutting out a block of agar plate culture and placing it face-to-face on a new plate to permit migration of the trophozoites away from the area of contact) has generally been used for isolation of amoebae. With the rich growth attained with the present method, the agar-block technique was unnecessary and isolation of the amoeba with or without a new bacterial associate could be done by the 'giant colony' technique (Oehler, 1916). In isolating the amoeba with a new bacterium, a small amount of a mixed culture growth was placed in the centre of an agar plate previously smeared with a suspension of the bacterium. When the new bacterium was edible, trophozoites migrated within a few hours after planting; when not edible, the trophozoites remained in close vicinity of the seeded area. Swarming of the original bacterial associate was prevented by using antibiotics in the inoculum. Penetration of the new bacterium-covered area may be as great as 25 mm. or more within 24 hr. Transfer of the growth 1 cm. or farther from the edge of the 'giant colony' usually produced a pure mixed culture of the amoeba and a new edible bacterium.

Cytological examination. The morphology and structure of *Naegleria gruberi* in the amoeba stage were studied with a phase-contrast microscope supplemented by Feulgen preparations stained by the method described by Singh (1952).

Survival tests of the trophozoites and cysts. The effects of drying on the survival of *Naegleria gruberi* were determined with growth material containing either trophozoites or cysts dried on slides. Water suspensions of the trophozoites or cysts were placed at −25° to −30° to test survival under freezing, and in a constant-temperature water bath to test resistance to heat. The effect of H^+ and OH^- ions on cyst survival was determined in HCl solution, HCl + Na acetate and $NaOH + Na_2HPO_4$ buffers and NaOH solutions. The test procedure used in studying cysts resistance to HOCl and I_2 was essentially the same as that used in several previous studies made on cysts of *Endamoeba histolytica* (Chang, 1944; 1955*a*).

RESULTS

Growth of Naegleria gruberi *on Czapek agar plates in association with* Proteus mirabilis

Growth curve. Repeated examination of large numbers of mixed plate cultures of *Naegleria gruberi* and *Proteus mirabilis* incubated at 25–27° and 37°, showed three distinct phases in the cultural life of the amoeba: lag growth, log growth, and growth encystment.

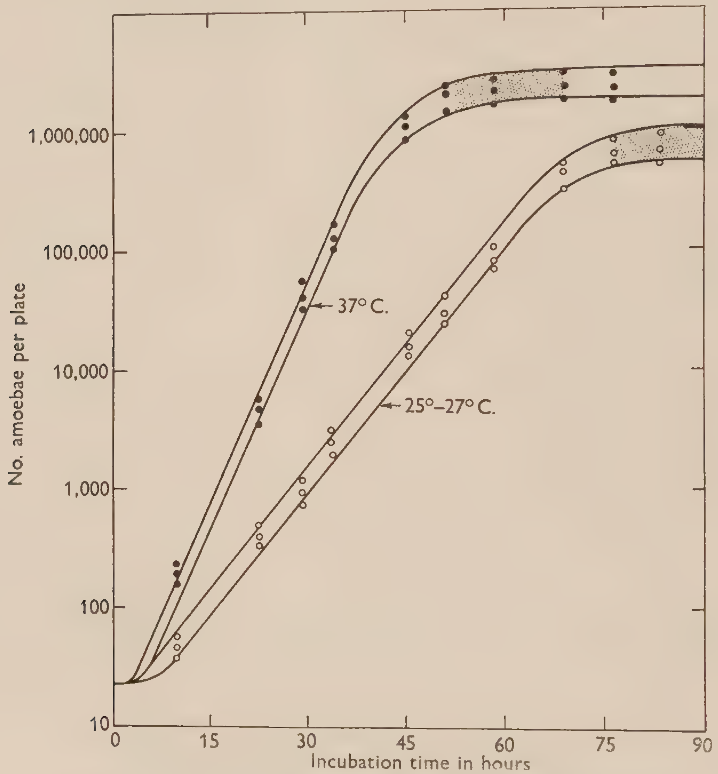

Fig. 1. Growth rate of *Naegleria gruberi* on Czapek agar plates (25–27° and 37°). ▨, Mass encystment.

Length of each growth phase and rate of amoeba growth during log growth phase. Each of 6 Czapek agar plates was seeded with a drop of a suspension of young trophozoites and *Proteus mirabilis*, diluted to contain not more than 50 amoebae/drop. The inoculum was smeared evenly with a sterile L-shaped glass rod over an area about 15 mm. in diameter. The exact number of amoebae on each plate was counted under a low power. Three plates were incubated with the lid uppermost at 37°, and three at 25–27°. Enumeration of amoebae was repeated every 2 hr. during the first 8 hr; then at suitable intervals during a 3-day period.

Figure 1 indicates that the lag phase lasted about 4 hr. at 25–27° and about 2 hr. at 37°, and the log growth phase about 60 and 40 hr., respectively, at these temperatures. A short period of slower growth followed the log growth phase and was interrupted by mass encystment, which lasted about 16 hr. Multiplication of the few remaining trophozoites contributed little to the total population, which remained more or less stationary during this phase. Later observations on tube cultures maintained on buffered sucrose nitrate agar slopes showed that the log growth and growth-encystment phases were not sharply demarcated and that, after 3–4 days of incubation, encystment and multiplication of trophozoites proceeded side by side in the mucoid deposit for several weeks.

Using conventional equations for computing the growth rate, *k*, and the generation time, *g*, the values of *k* (/hr.) and *g* (hr.) for the growth of *Naegleria gruberi* under these cultural conditions was found to be 0·68 and 4·5 at 25° and 1·21 and 2·5 at 37°. These values indicate that a temperature rise of 10° resulted in almost doubling the growth rate. However, this amoeba is apparently normally adapted to room temperature, and prolonged cultivation at 37° caused such degenerative changes as the appearance of giant trophozoites, temporary inability to transform into flagellates, etc.

Mass encystment. Initial encystment in a culture of *Naegleria gruberi* was always a mass phenomenon in which more than 90 % of the trophozoites were involved; it was usually observed in areas where the trophozoites grew into practically a single layer and the surface was relatively dry. Since the supply of bacterial food was still plentiful at this time, it appeared that the process was not stimulated by lack of food but rather by the drying and perhaps by other causes.

Growth of Naegleria gruberi *on other agar media in the presence of* Proteus mirabilis

Besides the buffered sucrose nitrate agar, other agar media were also used. The results are summarized in Table 1, which also includes the comparative results obtained with Czapek and buffered sucrose nitrate agar. The following results may be noted. First, the good growth of amoebae on Czapek agar plates but not on slopes can be attributed only to changes caused by the way the agar was poured. Since the mucoid growth was spread thin on plates but deposited at the bottom on the slope, and since the pH value of slope cultures was 0·3 unit lower than that of plates, it appeared that the short cultural life on slopes was a result of acid accumulation in the growth deposit in which the trophozoites were cut off from contact with air and were more affected by the pH value than those on plates. Examination of additional slope cultures during the first 24 hr. of incubation showed that practically all the trophozoites were washed to the bottom by the increasing amount of mucoid material produced by bacterial growth.

Secondly, the very poor growth of the bacterial associate in buffered lactose (or maltose) nitrate agar, due apparently to its inability to utilize these disaccharides, was accompanied by equally poor growth and early encystment of the amoeba. This observation shows that the amoeba feeds on bacteria and suggests that encystment is stimulated by a lack of food.

Table 1. *Growth of* Naegleria gruberi *in association with* Proteus mirabilis *on various agar media incubated at 25–27°*

B.S.N. = buffered sucrose nitrate agar; B.L.N. = buffered lactose nitrate agar; B.M.N. = buffered maltose nitrate agar; B.G.N. = buffered glucose nitrate agar; B.S.T. = buffered sucrose tryptose agar; B.T. = buffered tryptose agar.

			Slope		Plates	
Agar medium	Incubation time (hr.)	Growth of bacterium	Av. no. amoebae/ drop deposit	pH	Av. no. amoebae/ micro. field	pH
Czapek	24	Rich	Less than 100; motile	5·1	100	5·4
	48	Very rich	All non-motile	5·2	2500	5·5
B.S.N.	24	Rich	30,000	6·4	150	6·6
	48	Very rich	200,000	6·6	3000	6·7
B.L.N.	24	Very poor	No deposit, some growth on surface	7·3	10	7·4
	48	Very poor	Encystment	7·3	Encystment	7·4
B.M.N.	24	Very poor	Same as on B.L.N.	7·3	10	7·3
	48	Very poor	Encystment	7·3	Encystment	7·3
B.G.N.	24	Rich	10,000	5·7	100	6·0
	48	Very rich	50,000	5·9	1000	6·1
B.S.T.	24	Rich	40,000	6·4	250	6·5
	48	Very rich	300,000	6·5	3500	6·6
B.T.	24	Rich	4,000	7·0	50	7·1
	48	Very rich	50,000	7·0	600	7·1
Tryptose	24	Rich	Less than 100	6·2	10	6·3
	48	Very rich	Few dead ones	6·4	Mostly dead	6·4
Nutrient	24	Rich	Few dead ones	6·6	10	6·6
	48	Very rich	Few dead ones	6·5	Mostly dead	6·5

Thirdly, the poorer growth of the amoeba on buffered glucose nitrate agar, on which the bacterial associate flourished, coupled with a more acid pH value than that given on buffered sucrose nitrate agar, indicates that the more rapid decomposition of glucose than of sucrose resulted in a degree of acid accumulation less favourable for amoeboid growth. The rapid decomposition may also have depleted the carbohydrate supply, thus slowing the rate of multiplication.

Fourthly, the poorer amoeboid growth on buffered tryptose agar than on buffered sucrose tryptose agar, with an equally plentiful bacterial food supply, indicates that the addition of sugar increased the rate of multiplication.

Fifthly, the short cultural life of the amoeba on tryptose and on nutrient agar, on which the bacterial associate grew very well, can be attributed to the high concentration of tryptose, or the peptone and meat extract. This assumption was confirmed when a fairly good amoeboid growth was obtained on tryptose agar in which the tryptose was decreased from 2 to 0·2 %, and on nutrient agar in which the peptone and meat extract were both decreased to 0·1 %.

Growth of Naegleria gruberi *in association with* Proteus mirabilis *in various media incubated at* 25–27°

Amoeboid growth in liquid media in association with *Proteus mirabilis* was studied. The results (Table 2) agreed with those in Table 1 in that tubed media tended to accumulate acid at the bottom, thus reducing or even preventing multiplication. They also indicate that the liquid media did not support growth as well as the agars. Other observations, with micro-cultures, showed that the poor growth in liquid media was due to the difficulty of the trophozoites in feeding on freely suspended bacteria, whereas on agars the trophozoites are immediately surrounded by bacteria.

Table 2. *Growth of* Naegleria gruberi *in association with* Proteus mirabilis *in various fluid media incubated at* 25–27°

B.S.N. = buffered sucrose nitrate solution; B.S.T. = buffered sucrose tryptose broth; B.T. = buffered tryptose broth.

		Tube cultures			Petri dish cultures	
			pH			
Medium	Incubation time (hr)	Av. no. amoebae/ drop fluid	Top	Bottom	Av. no. amoebae/ micro. field	pH
Czapek solutions*	Start	50	7·2	7·2	4	7·2
	24	All non-motile	5·2	4·9	All non-motile	5·2
	48	All dead	5·3	5·0	All dead	5·4
B.S.N.*	Start	50	7·5	7·5	5	7·5
	24	All non-motile	5·8	5·5	Less then 10	5·9
	48	All dead	5·8	5·6	All encysted	6·0
B.S.T.†	Start	50	7·6	7·6	5	7·6
	24	About 100	6·2	5·9	25	6·2
	48	All encysted	6·3	6·1	50; 30 % cysts	6·4
B.T.†	Start	50	7·6	7·6	5	7·6
	24	Less than 100	7·4	7·4	10	7·4
	48	All encysted	7·5	7·5	20; 50 % cysts	7·5
Tryptose broth†	Start	50	7·1	7·1	5	7·1
	24	All non-motile	6·9	6·8	All non-motile	6·9
	48	Few cysts left	6·8	6·8	Few cysts left	6·8
Nutrient†	Start	50	6·7	6·7	5	6·7
	24	All non-motile	6·6	6·5	All non-motile	6·6
	48	Few cysts left	6·6	6·5	Few cysts left	6·5

* Good growth of the bacterium-associate judged by the amount of turbidity.

† Excellent growth of the bacterium-associate.

It appeared that low pH values were more harmful to amoebae in liquid media than on agar plate cultures. Lowering the pH value to 5·4 in Czapek solution dispensed in Petri dishes resulted in death, whereas a similar pH lowering exerted no harmful effect on Czapek agar plate cultures. Other observations with trophozoites suspended in water buffered with phosphates at low pH values (5·2–7·0) revealed that trophozoites become non-motile and non-feeding, and survived not more than 16 hr. at pH 5·6 or less. pH values of 5·6 and less of the liquid media interfered with the physiological activities of the trophozoites, such as removal of CO_2 from the organism, whereas on agar plates the pH value seems less likely to interfere with the gas exchange as the trophozoites are in partial contact with the air.

Redox potential and pH *changes in mixed cultures of* Naegleria gruberi *and* Proteus mirabilis

Since buffered sucrose tryptose broth of the liquid media studied was found to produce the best amoeboid growth this medium was used in studying potential and pH changes in a mixed culture of these two organisms (Chang, 1946). E_h and pH values were obtained for each of three replicate cultures, maintained at 25–27° and 37°. The E_h value dropped steadily and sharply during the first 8 hr at 25–27° and the first 4 hr. at 37° until a value of −110 to −130 mV was reached. After this decrease, the potential rose steadily to reach +180 to +200 mV at the end of 24 hr. of incubation. Only a 20 mV increase in potential appeared during the remaining 5 days, to give an E_h value of +200 to +220 mV at the end of the 6th day. The pH value also dropped steadily to 6·2 in 12 hr. at 25–27° and in 9 hr at 37°. After the initial decrease there was a slow but steady rise to a value of 7·1 at the end of 6 days. Changes in these cultures would have been considerably greater if the medium had not been so strongly buffered. These results indicate that the mixed culture was, except for the first 8–12 hr. of incubation, an aerobic system, and that decomposition of sucrose with accumulation of acid was most rapid during the first 12 hr. at 37° and the first 24 hr. at 25–27°. The redox potential value would still be relatively high even after correction for lowering of the pH value.

Role of the bacterial associate in the cultivation of Naegleria gruberi

Attempts were made to replace *Proteus mirabilis* in the mixed culture by other bacteria. With the richness of amoeboid growth attained in the cultures, the use of the ‘agar block’ technique was unnecessary and the establishment of a new association could be made by smearing a rich suspension of the new bacterium over the entire surface of a plate of suitable agar medium and placing a small amount of growth material from the old culture at the centre (Oehler, 1916). However, in repeated attempts to replace *P. mirabilis* by *Escherichia coli* or *Aerobacter aerogenes*, swarming of the former and the inhibitory effect of antibiotics on the substitute bacterium in the vicinity of the inoculum resulted in failures.

Replacement by a Flavobacterium *sp.* Having found by accident that the *Flavobacterium* sp. isolated from Ohio River water exhibited some antibiotic effect on *Proteus mirabilis* and prevented swarming, an effort was made to replace the latter by the former in amoeba cultures. With Oehler’s technique, replacement was successful at the first attempt made on buffered sucrose tryptose agar plates. However, during the early subcultures, some difficulty arose from the toxic effect of the yellow pigment, and only those trophozoites in the periphery of the bacterial colonies survived and underwent encystment. A preliminary study with an acetone extract of the flavobacterium showed that the pigment inhibited the feeding of the trophozoites on bacteria. This antibiotic effect was temporary, and after four to six subcultures the amoebae apparently developed tolerance to the pigment and grew into cultures as rich as in the presence of *Proteus mirabilis*. While the general growth pattern was essentially the same, the log-growth phase was shortened by 12–24 hr. by early mass encystment. In the new association, the trophozoites were found frequently in areas without bacterial growth, a phenomenon not observed in association with *P. mirabilis*.

Replacement by other bacteria. With mixed cultures of *Naegleria gruberi* and the *Flavobacterium* sp., replacement of the bacterial associate was simple provided the replacement bacteria were edible. Up to the present, pure mixed cultures of the amoeba have been maintained with *Escherichia coli*, *Aerobacter aerogenes*, on buffered sucrose tryptose agar, and with *Salmonella typhosa*, *S. paratyphi*, *S. schottmuelleri*, *S. enteritidis*, *S. cholerae-suis*, *S. typhimurium*, *S. meleagridis*, *S. pullorum*, *Shigella sonnei* and *Sh. dysenteriae* II on buffered sucrose glucose tryptose agar; but failures were encountered with *Bacillus subtilis*, *Pseudomonas pyocyanea*, and *Sarcina lutea*, attributable, with the last two organisms, to the toxic effect of their pigments.

Growth of Naegleria gruberi *in the absence of living bacteria.* Numerous plates were prepared on which the trophozoites were free from bacteria. These trophozoites invariably and rapidly underwent encystment, and the cysts failed to excyst unless living edible bacteria were added. Incorporation of vitamins, amino acids and growth factors in the medium failed to alter the fate of these pure cultures, reflecting the complexity of the nutritional requirement. Addition of edible species of bacteria used in the present study but killed by heating at 60° for 60 min., produced very poor growth. It seems that a thermolabile growth-promoting factor present in the living bacteria is required.

Some cytological observations on Naegleria gruberi

Size. The size of this amoeba is noticeably influenced by its rate of multiplication, which, in turn, is influenced by food supply. Under cultural conditions in which the trophozoites were supplied with unlimited numbers of bacteria and sufficient carbohydrate, the trophozoites multiplied at maximum rate and the size of the rounded trophozoites at the end of the log growth phase was 18μ. average diameter, whereas under natural conditions in raw river water, the average diameter was 22μ. The size of the cysts depended entirely on the size of the trophozoites undergoing encystment. In general, there is a decrease to three-quarters of the diameter at encystment.

Appearance of cysts. The cysts formed in these cultures were smoother in outline than those illustrated by Singh (1952). Occasionally spindle-shaped cysts were seen in isolated areas of plate cultures. The outer wall of cysts formed on *Proteus mirabilis* was thicker than those formed with other species of bacteria and had one or two but rarely three pores, filled apparently with a hyaline-like substance (Pl. 1, fig. 1) which were found to provide exit for the metacystic amoeba.

Resting nucleus. The trophozoites were usually uninucleate; the frequency of occurrence of binucleate amoebae (Pl. 1, fig. 2) was *c.* 1 in 300. Uninucleate and binucleate cysts were formed from trophozoites which had the corresponding number of nuclei; amoebae with more than two nuclei were not observed. The newly excysted amoebae had the same number of nuclei as their mother cysts.

Because of its fluidity, the shape of the nucleus changed as the endoplasm flowed from one direction to another during the motion of a trophozoite; it was spherical only when the amoeba was stationary or in the cystic stage. These observations apply also to the karyosome (Pl. 1, figs. 3, 4).

In confirmation of the observations of Singh (1952), the karyosome was Feulgen-negative, sometimes showing one or two areas of lighter intensity which appeared unstained by the light green counter-stain of the Feulgen technique. The nuclear membrane was a delicate structure visible only when the chromatin granules were slightly out of focus (Pl. 1, fig. 4). A single layer of short rod-shaped chromatin granules lay directly beneath and perpendicular to the nuclear membrane. They were more easily seen in cysts than in moving trophozoites (Pl. 1, figs. 1, 2). Thread-like structures radiating from the karyosome as described by Rafalko (1947) and by Singh (1952) were not encountered. Short chromatin rods appeared as chromatin patches (Singh, 1952) when not in sharp focus.

The nucleus in mitosis. The principal nuclear changes observed during mitosis in *Naegleria gruberi* have been so fully described and illustrated by Rafalko (1947) and Singh (1952) that only the origin and fate of the 'interzonal body' and the polar caps are described here. When the karyosome divides into two polar masses in the prophase, a piece drops out. This piece can be seen through the metaphase and develops into the 'interzonal body' in the anaphase, joining the polar mass in the telophase to form the karyosome of the daughter nucleus. The achromatic polar caps described by Ford (1914) and confirmed by Rafalko (1947) and Singh (1952) derive also from the karyosome. During the early part of the metaphase, a small piece of the polar mass protrudes toward the nuclear membrane and develops into a cap. During the latter part of the anaphase, at about the same time that the 'interzonal body' divides, each polar cap retreats towards the polar mass and fuses with it.

Contrary to Rafalko (1947), and in agreement with Singh (1952), no centrioles were seen at the apex of the polar cap during nuclear division in *Naegleria gruberi*. In numerous cases of nuclear division no suggestion of centrioles was observed. The observation of Wilson (1916) regarding endogenous budding in *N. gruberi* was not confirmed.

Effect of physical and chemical agents on the survival of trophozoites and/or cysts of Naegleria gruberi

The results obtained on the effects of drying, freezing and heat on the survival of trophozoites and cysts of the amoeba are summarized in Table 3. Direct microscopic examination of the slides prepared in the drying experiment revealed that the cysts, dehydrated beyond recognition as soon as the water

Table 3. *Effects of drying, freezing and heat on the survival of trophozoites and cysts of* Naegleria gruberi

		Time for			
		Trophozoites		Cysts	
Physical agent	Testing conditions	Last pos. cultures	1st neg. cultures	Last pos. cultures	1st neg. cultures
Drying	On slides at 25–27°	0 min.	15 sec.	23 months	Unknown
Freezing	At $-25°$ to $-30°$	40 min.	45 min.	$3\frac{1}{2}$ months	4 months
Heating to:					
47°	In water at pH 6·8	30 min.	35 min.	—	—
50°	In water at pH 6·8	1 min.	2 min.	30 min.	35 min.
60°	In water at pH 6·8	—	—	1 min.	2 min.

film disappeared, resumed their normal appearance after flooding with water. The trophozoites, on the other hand, appeared dull, granular and shrunk on re-hydration, even when water was added within a few seconds after drying.

The results obtained in experiments on destruction of cysts by H^+ and OH^- ion, HOCl and I_2 are shown in Table 4.

Table 4. *Resistance of cysts of* Naegleria gruberi *to* H^+ *and* OH^- *ion,* HOCl *and* I_2 *at* 25–27°

	Concentration		Time for	
Chemical agent	Initial	Residual	Last pos. cultures	1st neg cultures
H^+	—	≡pH 0·5	$2\frac{1}{4}$ hr.	Undetermined
OH^-	—	≡pH 14·0	5 min.	10 min.
HOCl*	1·8 p.p.m.	1·5 p.p.m.	30 min.	35 min.
	4·0 p.p.m.	3·5 p.p.m.	10 min.	15 min.
I_2	2·5 p.p.m.	2·0 p.p.m.	30 min.	35 min.
	4·5 p.p.m.	4·0 p.p.m.	10 min.	15 min.

* Expressed as titratable chlorine.

DISCUSSION

The study of the nutritional requirements of *Naegleria gruberi* was handicapped by the need for edible living bacteria, but this need reflects the nutritional complexity of the amoeba. In practice, this method of cultivation offers a reproducible procedure by which the cultural characteristics of the amoeba can be studied. The growth rate of *N. gruberi* at room temperature determined in the present work is not significantly different from that of *Endamoeba histolytica* at 37° as computed from data of Balamuth & Howard (1946) and of Phillips & Rees (1950). On the other hand, it is considerably higher than that of *Tetrahymena geleii* at 25° (Kidder & Dewey, 1949) and of haemoflagellates at 22·5–32° (Chang & Negherbon, 1947).

The selectivity of *Naegleria gruberi* for species of bacteria as food is a subject of considerable interest. Singh (1941, 1942*a*, *b*, 1945) showed that free-living amoebae fed selectively on various bacteria species, generally excepting those which produce red, violet, blue, green or fluorescent pigments. In the present work, it was found that all species of *Salmonella* and *Shigella* tested, *Escherichia coli*, *Aerobacter aerogenes*, *Proteus mirabilis*, and a *Flavobacterium* sp. were edible but that the last one exhibited a temporary antibiotic effect. *Bacillus subtilis*, *Sarcina lutea*, and *Pseudomonas pyocyanea* were inedible, the last two because of the toxic effect of their pigments toward which the amoeba did not acquire tolerance as it had towards the yellow pigment of the flavobacterium. The edibility of Salmonella and Shigella organisms by amoebae has some sanitary significance. It indicates that in sewage treatment, as well as in natural purification of polluted water, this method of feeding may be responsible, at least in part, for the diminution in numbers of pathogenic bacteria. In this connexion Staal (1954) reported the feeding of Paramecium on *E. coli*, *Micrococcus pyogenes* var. *aureus*, *S. typhimurium*, *S. enteritidis*, and *B. subtilis*, the harmful effect of *E. coli* and *S. typhimurium* on the predator, and that as many as 10,000 bacteria may be consumed/Paramecium/hr. Since Staal used killed organisms stained with a dye to facilitate observation, his results may not be applicable to protozoa feeding on living bacteria. In practice, both observations indicate the presence in sewage and natural waters of predators which would substantially decrease the numbers of enteric pathogens. Results obtained on amoeboid growth with *P. mirabilis* on various agar media strongly indicate that while the amoeba relies on the bacteria for food, utilization of this food is improved by the presence of a carbohydrate.

While observations on redox potential changes in mixed cultures of *Naegleria gruberi* and *Proteus mirabilis* indicate a relatively aerobic system, they do not rule out amoeboid growth under an anaerobic system. Rafalko (1947) claimed that *N. gruberi* grew in faecal suspension but made no mention of degree of growth nor of the period for which his cultures were maintained.

The practically instant death of trophozoites and the extremely long survival of cysts of *Naegleria gruberi* under drying conditions indicate some fundamental difference in the organization of the protoplasm as between the vegetative and resting forms. Since the cysts rapidly restored their normal appearance upon re-hydration while the trophozoites showed signs of permanent changes, it appears that the amoeba undergoes a reorganization of the protoplasm in encystment which enables the cysts to survive dehydration. This ability shows a difference between cysts of *N. gruberi* and those of *Endamoeba histolytica*, since the latter die almost instantaneously upon drying (Chang, 1943).

Results on the survival of trophozoites and cysts of *Naegleria gruberi* under freezing conditions compare with those under drying. It was stated (Chang, 1955*b*) that freezing kills cysts of *Endamoeba histolytica* by denaturation of protoplasmic proteins through crystallization of their water content. If this hypothesis is applicable to *N. gruberi*, one would expect rather rapid death of trophozoites and long survival of cysts on freezing. As expected, the survival time of trophozoites of *N. gruberi* at −25° to −30° was about 40 min., the time required for freezing the suspension. However, the 3- to 4-month survival of the cysts when frozen as against the 23-month or longer survival when dried remains unexplained. The data on the destruction of cysts by H^+ and OH^- ion, HOCl and I_2 obtained here strongly indicate that while these cysts survive much longer periods in a dehydrated state than those of *Endamoeba histolytica*, they are only slightly more resistant to these chemical agents than are the latter (Fair, Chang & Morris, 1945; Fair, Morris & Chang, 1947; Chang & Morris, 1953). Hence, it seems that cysts of *N gruberi* might be used advantageously as a substitute for those of *E. histolytica* in cyst-destruction studies.

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EXPLANATION OF PLATE

Phase-contrast micrographs of trophozoites and cysts of *Naegleria gruberi.*

Fig. 1. A cyst showing the outer wall and its pore, and chromatin rods arranged perpendicular to nuclear membrane.

Fig. 2. A binucleate cyst.

Fig. 3. A trophozoite showing the nucleus.

Fig. 4. A dividing trophozoite showing the nuclear membrane or chromatin and the karyosome of each nucleus.

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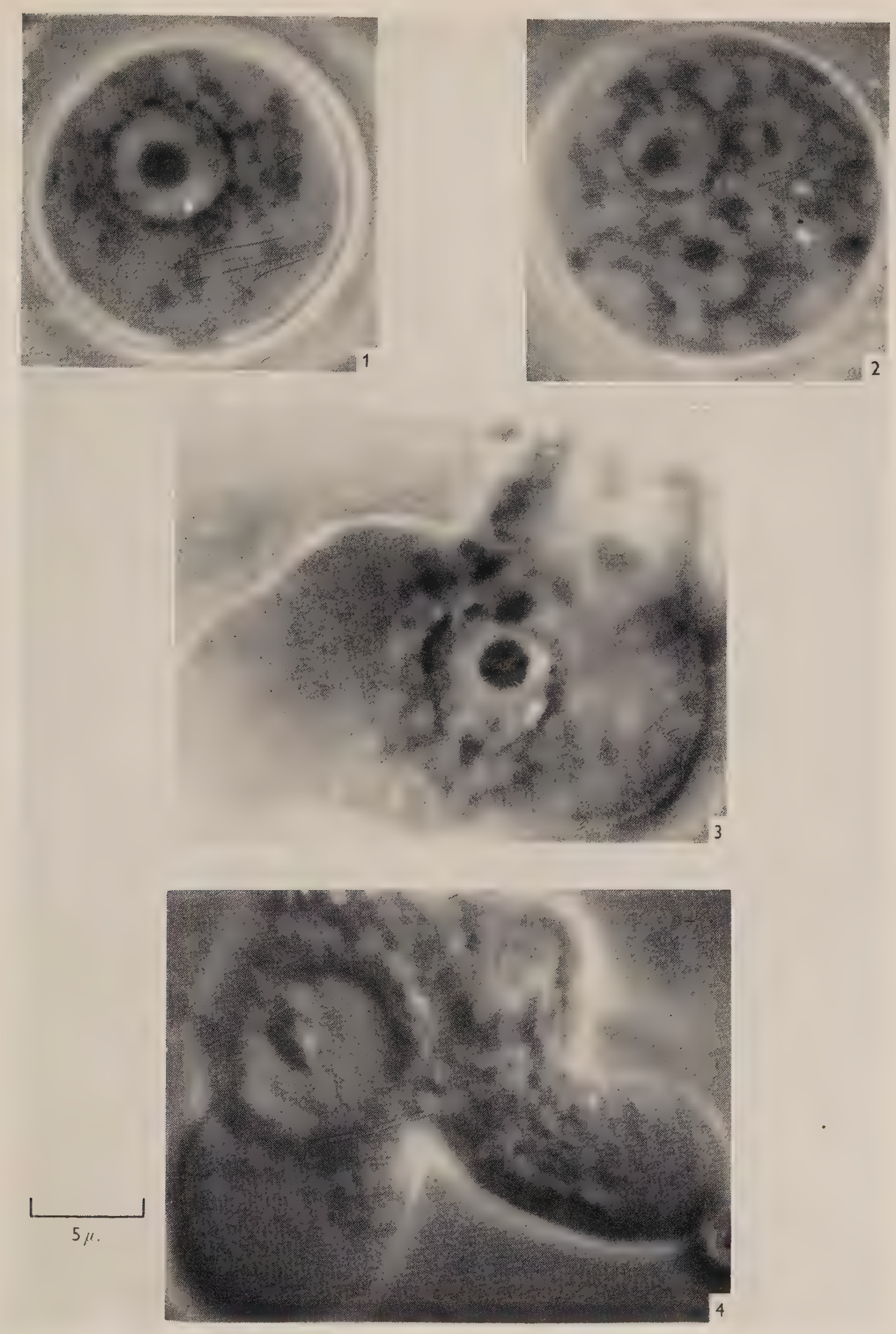

S. L. CHANG—AMOEBA STAGE OF *NAEGLERIA GRUBERI*. PLATE 1

(*Facing p.* 578)

CHANG, S. L. (1958). *J. gen. Microbiol.* 18, 579–585

Cytological and Ecological Observations on the Flagellate Transformation of *Naegleria gruberi*

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SUMMARY: Phase-contrast microscopic examination of *Naegleria gruberi* (Schardinger) during flagellate transformation and reversion revealed the following cytological features. Flagella were formed by filamentous extension from endoplasmic protrusion. One, two or three pairs of flagella were formed from a single protrusion. Occasionally a second protrusion gave rise to the 3rd pair of flagella. A chromatin body of cytoplasmic origin was always present at the base of the protrusion or of the flagella, and is believed to be the parabasal body. Occasionally one chromatin body was seen at the base of each pair of flagella. Alternating bands of light and dark areas were seen in the flagella soon after they were formed and persisted throughout the flagellate stage. Reversion from flagellate to amoeba stage was accomplished by absorption of the flagella, the shedding of one or more flagella and the absorption of the rest, or by casting-off a small part of the body to which the flagella were attached. The following effects of environmental factors on flagellate transformation were observed. As many as 35 % of the trophozoites underwent flagellate transformation in water when they were in metacystic stage or were harvested from plate cultures up to 12 hr. of incubation at 25–27°; as the cultural age advanced, the % amoebae undergoing transformation became progressively lower. Prolonged cultivation in a liquid medium at 25–27°, or on an agar medium at 37°, resulted in temporary loss of ability to form flagellates. The freezing of trophozoites up to 40 min. and of cysts up to 1 week at $-25°$ to $-30°$ stimulated flagellate transformation. An increase in water temperature from 25–27° to 37° or decrease to 8–10° suppressed the transformation, but after the flagellates were formed, the former temperature change shortened while the latter lengthened duration of the flagellate stage. Freezing of the amoebae in the flagellate stage at $-25°$ to $-30°$ exerted no adverse effect on their return to amoeboid form when the freezing time did not exceed 30 min. Cultures developed from single organisms isolated in the flagellate stage yielded amoebae showing flagellate transformation characteristics similar to those yielded by ordinary cultures.

Detailed study of the transformation of *Naegleria gruberi* (Schardinger) from amoebae to flagellates and vice versa is yet to be made. With the strain of *N. gruberi* maintained in rich permanent cultures as described in the preceding paper (Chang, 1958), observations were made on the cytology of flagellate transformation and reversion to amoebae with a phase-contrast microscope, and also of the effects of various environmental factors on this morphological transformation.

METHODS

Pure mixed cultures of *Naegleria gruberi* and *Proteus mirabilis* were maintained at room temperature on buffered sucrose nitrate agar (Chang, 1958). For this study, the mucoid growth from a young plate culture was suspended in a small amount of distilled water and examined with a phase-contrast microscope. To immobilize the flagellates for photomicrography, both cold and heat proved unsatisfactory, but a concentration of *c.* $5\mu g$. $AgNO_3$/ml. in the suspension successfully immobilized the flagellates with fewer protoplasmic changes than other chemicals. In order to observe the cytological characteristics of the flagellate forms that were obliterated by the $AgNO_3$ treatment, photomicrographs were taken of those about to undergo reversion and moving slowly enough to permit photomicrography. Because of the limited amount of light transmitted by the phase-contrast microscope, exposure times of a few seconds were required and some blurring was unavoidable. To develop pure-line cultures of *N. gruberi* from single organisms in the flagellate stage, flagellates induced in sterile distilled water were fished out singly with micropipettes and placed on agar plates pre-seeded with *P. mirabilis*. All inoculated plates were thoroughly examined under a low power, and only those with a single flagellate were kept at 25–27° for development of positive cultures.

RESULTS

Cytology of the flagellate transformation

At the start of the flagellate transformation, the trophozoites formed pseudopodia and retained motility, but at the same time they protruded an endoplasmic mass. After the protrusion reached a certain size, it constricted at the base. The apparent homogeneous mass formed a pair of flagella by extending two long filamentous processes, one after the other (Pl. 1, fig. 1). In the formation of two or three pairs of flagella, corresponding processes were extended from the protruded mass. Occasionally a second protrusion formed near the first one, for the formation of the 3rd pair of flagella. Formation of more than three pairs of flagella was not observed. The report of Wenyon (1926) that one, two, or three pairs of flagella were formed in individuals having the corresponding number of nuclei was not confirmed. In all cases of flagellate transformation observed, the trophozoites were exclusively uninucleate.

While the flagella were being formed, a chromatin body of cytoplasmic origin was always present at the base of the protrusion. After the flagella were formed, this chromatin body was located at the base of the flagella and is believed to be the basal body. When more than one pair of flagella were formed, a basal body might be present at the base of each pair (Pl. 1, fig. 2). The statements of Alexieff (1912) and of Wilson (1916) that one blepharoplast is present at the base of each flagellum, that it is of karyosome origin and is connected to the flagellum by a rhizoplast, were not confirmed. Soon after the flagella were formed, there appeared to be a short period during which the endoplasm in each flagellum underwent rearrangement to form alternating light and dark bands (Pl. 1, figs. 3, 4), and the ectoplasm formed a thin layer beneath the cytoplasmic membrane. The alternating bands are believed to be of sol and gel and the peripheral arrangement of the ectoplasm to be responsible for the cell wall-like appearance in the flagellate forms (Pl. 2, figs. 5–7).

Cytology of the flagellate forms

The alternating light and dark bands in the flagella persisted throughout the flagellate stage. As soon as the flagella exhibited movement, the body became pear-shaped with the flagella located at the pointed end (Pl. 2, figs. 5–7). At this stage, the amoebae lost their identity and looked in every respect like 'flagellates'. Movement of the flagella, whether of one, two or three pairs, was circular and so well co-ordinated that the organisms were propelled forward in a straight course. When a turn was made, the posterior end of the organism tilted toward the direction of the turn.

Although the appearance of the alternating bands was abolished by the use of $AgNO_3$, the flagella, the basal body and the nucleus were easily recognizable. The basal body at the base of the flagella was very close to the cytoplasmic membrane (Pl. 1, fig. 2; Pl. 2, fig. 5). The nucleus might be located in any part of the body, although most frequently it was in the anterior one-third (Pl. 2, figs. 5–7). The large contractile vacuole situated near the posterior end, as described by Singh (1952), was frequently absent. When present, it might be located in any part of the posterior half. When two pairs of flagella developed, they were arranged very close to each other (Pl. 2, fig. 7). In some flagellate forms there appeared to be a cytostome-like structure, as suggested by Wenyon (1926), along one side of the anterior end (Pl. 2, fig. 6). Repeated examination of a large number of these forms revealed that this groove-like appearance was caused by the slight twisting of the anterior end as the organisms were propelled forward in a rotary motion.

Abnormal flagellate forms

During the flagellate transformation, there were significant numbers of trophozoites which showed such abnormal development that they failed to complete the transformation. These abnormal forms were generally not recognized in ordinary microscopic examination. In some cases, elongation of the endoplasmic protrusion resulted in the formation of a long filamentous process with a substantial piece of endoplasm remaining at the end, which might or might not show signs of bifurcation. Some trophozoites developed structures like tree-branches from the protruded mass; some formed numerous short processes without flagella formation. Still others formed multiple protrusions, some of which extruded long processes while others became detached. The first three types of abnormal forms usually reverted to the amoeba stage; the last types often disintegrated.

Cytology of reversion

Because of the non-feeding nature of the flagellate forms, the ceaseless motion apparently results in a rapid exhaustion of stored food. It is believed that return to the amoeba forms is stimulated as this stored food is consumed. Generally speaking, after 2–3 hr. of continuous movement, the flagellates frequently showed a change of body shape. Since the ectoplasm is distributed in a thin layer under the cytoplasmic membrane, bulges contain mostly endoplasm. As a result of changes in body shape, forward movement tended to be irregular rather than straight. Soon the organisms became more and more amoeboid in shape, with movement becoming slower and more irregular. Eventually, the shapes became so altered that the propelling action of the flagella resulted in a rotary motion without much change in position. Finally movement stopped, although the flagella might continue to show some weak circular motion for a few seconds. As soon as they completely stopped movement, the alternating light and dark bands disappeared, giving further evidence that they were intimately associated with movement.

There appears to be at least three ways by which flagellate forms revert to amoeboid forms. In the majority of cases, reversion was accomplished by a continuous shortening of the flagella, resulting in the formation of beaded processes with progressively fewer but larger beads until the whole process was absorbed by the body (Pl. 2, fig. 8). Rafalko (1947) considered this the only means of amoeba reversion. Less frequently, reversion was achieved by dropping parts of one or more flagella and absorbing the rest. In a very few individuals, the return was completed by the formation of a constriction near the base of a flagellum, which eventually separated the flagellum and a small piece of endoplasm from the rest of the organism (Pl. 2, fig. 9). Following the first two types of reversion, the amoebae invariably encysted. The fate of the amoebae after the last type of reversion is unknown.

Effects of environmental factors on flagellate transformation

Cultural age. By using newly excysted amoebae and trophozoites harvested from plate cultures of different incubation periods, the influence of the cultural age on the flagellate transformation was studied; the results obtained are shown in Table 1.

Table 1. *Effect of incubation time of plate cultures of* Naegleria gruberi *on the flagellate transformation in water*

Cultural age (hr.)	Flagellate transformation (%)
Metacystic amoeba	30–35
4– 8	25–35
12–14	20–30
24–32	10–15
48–56	5–10
60–72	1– 5
80–96	<1

Cultivation in a fluid medium. Trophozoites harvested from cultures in buffered sucrose tryptose broth (Chang, 1958) after different incubation periods and concentrated by low-speed centrifugation, were suspended in distilled water to induce flagellate transformation. The results in repeated experiments showed that the amoebae lost ability to form flagellates after 48 hr. or longer in the broth. By transferring amoebae from the liquid medium to the agar medium, flagellate transformation was regained after two or more subcultures; but the longer the organism was maintained in broth cultures, the more subcultures on solid medium were required to regain the ability to transform. For example, five to six plate subcultures (6–8 weeks) were required to retain this ability after the amoeba had remained in broth cultures for 4–5 weeks.

Cultivation on an agar medium at 37°. Repeated attempts were made to induce flagellate transformation of newly excysted amoebae and young trophozoites harvested from plate cultures maintained at 37°. Results indicated that the amoeba lost its flagellate-forming ability after two or more subcultures (2 weeks or longer) at 37°. This phenomenon was temporary and the ability was regained by the amoeba after several transfers at 25–27°. It was not ascertained whether or not longer cultivation at 37° would result in permanent loss of this ability.

Freezing at −25° *to* −30°. Water suspensions of trophozoites of various cultural ages were frozen at −25° to −30° shortly after preparation to avoid flagellate transformation. Samples were removed one at a time, thawed, and examined for flagellate forms. The results revealed that freezing for 10–40 min. increased flagellate transformation by about 15% over that observed with unfrozen amoebae of the same cultural age; freezing for more than 40 min. resulted in death.

The next step was to determine how freezing would effect survival of the flagellates and reversion to the amoeba forms. Water suspensions of trophozoites of *c.* 12 hr. cultural age were allowed to stand until a maximum number of flagellates was formed. These were then frozen, thawed, and examined for motility and reversion as described above. The results indicated that freezing at −25° to −30° up to 40 min. exerted no adverse effect on these activities. Like the trophozoites, the flagellate forms were killed when the freezing time was extended over 40 min.

It is interesting to note that while the flagellate transformation was never observed on agar plates in routine cultures, it was repeatedly noted during the first few hours of incubation on plates seeded with cysts which had previously been frozen at −25 to −30° from a few hours to about 1 week. Frequent examinations during the early hours of incubation revealed that many of the newly excysted amoebae underwent flagellate transformation soon after they emerged. The number of newly excysted amoebae undergoing transformation became progressively less as freezing time of the cysts was lengthened; none formed flagellates after >3 weeks of freezing.

Effect of temperature on flagellate transformation and duration of the flagellate stage

A water suspension of young trophozoites was dispensed in five Petri dishes and handled in the following manner: one was placed in a refrigerator at 8–10°, one in an incubator at 37°, and three were left at 25–27°. All dishes were examined at hourly intervals for flagellates. As soon as the number of flagellates reached the peak in the dishes left at 25–27° one dish was moved to the refrigerator, one to the 37° incubator, and the third remained at 25–27°. Examination of all dishes continued until the flagellates returned to amoebae.

In three replicate experiments it was found that storage of the trophozoites at 8–10° or at 37° suppressed the flagellate transformation, and that while most of the transformed organisms returned to amoebae within 6 hr. at 25–27°, the flagellate stage was lengthened to 8–10 hr. at 8–10° and was shortened to *c.* 2 hr. at 37°. Flagellate motility at 8–10° was noticeably slower and at 37° somewhat faster than at 25–27°.

Flagellate transformation of trophozoites from pure-line cultures developed from single flagellates

Of a total of ten isolations made in the flagellate stage, five developed positive amoeba cultures. These pure-line cultures resembled others in cultural appearance and in growth and encystation characteristics. By suspension in water they exhibited flagellate transformation activity identical with that of ordinary cultures.

DISCUSSION

The development of flagella is the most fascinating phenomenon in the whole process of flagellate transformation. Some internal rearrangement of the endoplasm must take place before the formation of the protrusion in order to provide homogeneous material for the formation of the flagella, and of a chromatin body of cytoplasmic origin for the formation of the basal body or bodies. The alternating light and dark bands in the flagella appear to be bands of sol and gel and are believed to be responsible for the motility of the flagella. A central contractile fibril has been demonstrated in the flagella of some large flagellates; however, the presence of such a fibril has not been demonstrated in smaller forms such as *Trichomonas* and *Chilomastix* spp. It would be interesting to determine whether the mechanism of flagella motility in these forms is similar to that of the transformed flagellates of *Naegleria gruberi*. In most true flagellates, the anterior end is the more blunt, enabling the organisms to swim with the least amount of hydraulic resistance. In the transformed flagellates of *N. gruberi*, the anterior end is more pointed, due to the lack of a sufficiently rigid wall around the organisms to maintain the typical 'fish' shape when the hydraulic pressure exerted at the anterior end of the moving organisms is greater than at the other points.

The observations obtained on the effects of various environmental factors on flagellate transformation of *Naegleria gruberi* justify the following general statements: (1) flagellate transformation is an activity of this flagellate amoeba, transmissible through its offspring on a fixed percentage basis; (2) the potentiality of flagellate transformation is at its peak in newly excysted amoebae and in very young trophozoites, decreasing steadily as the cultural age advances; trophozoites of very old cultures lose most of this potentiality; (3) the mechanism of flagellate transformation is probably triggered by hydration of a certain part of the cytoplasm of the amoeba and occurs when newly excysted amoebae or plate-grown trophozoites are suspended in water; this hydration effect, however, may be obliterated by growing the amoeba in a fluid medium or by prolonged culitvation at 37°; (4) freezing of trophozoites

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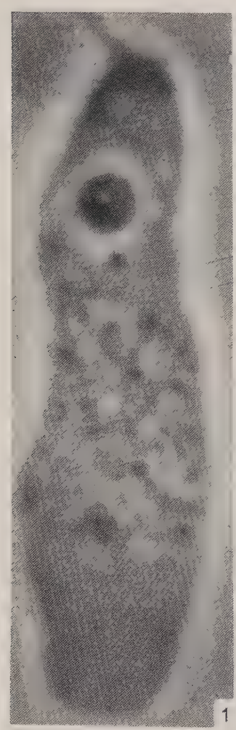
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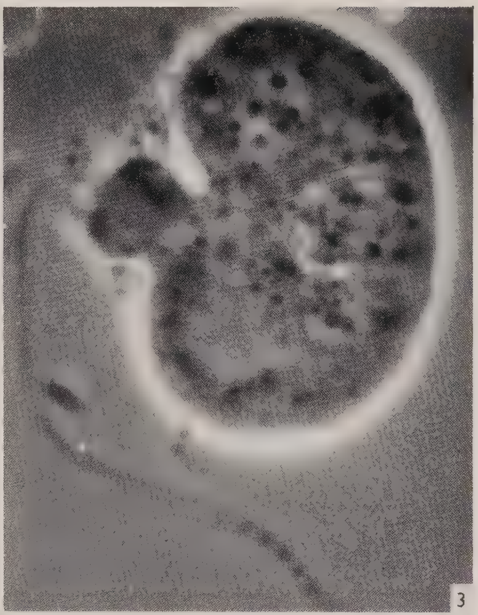
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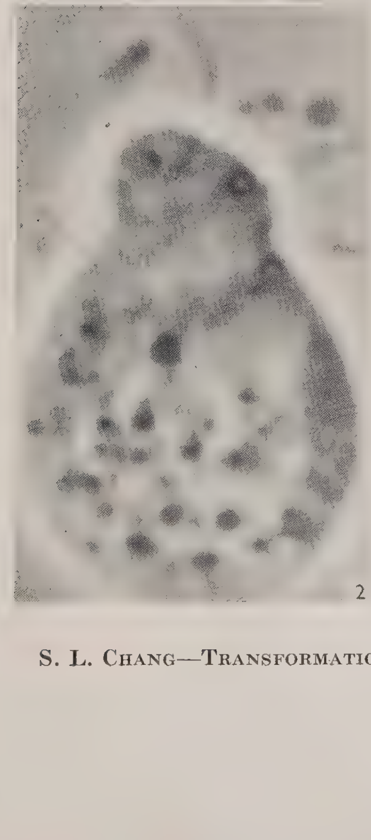
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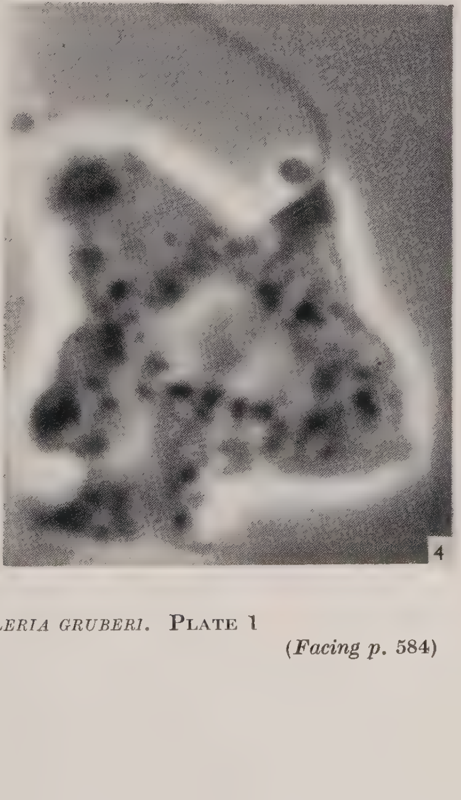
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S. L. CHANG—TRANSFORMATION OF *NAEGLERIA GRUBERI*. PLATE 1

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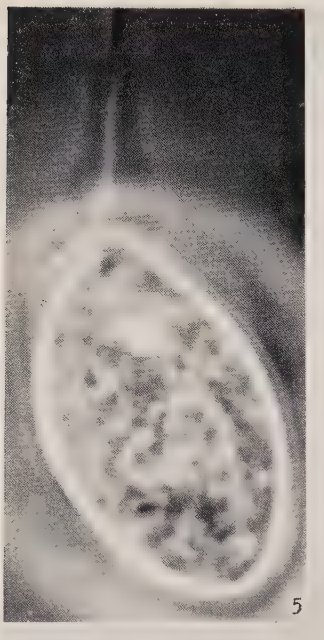

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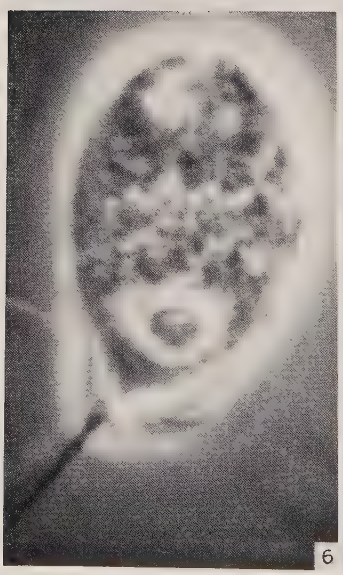

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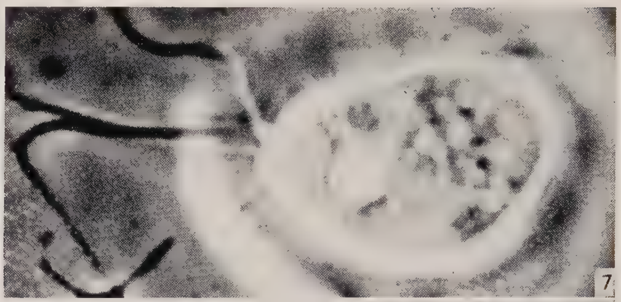

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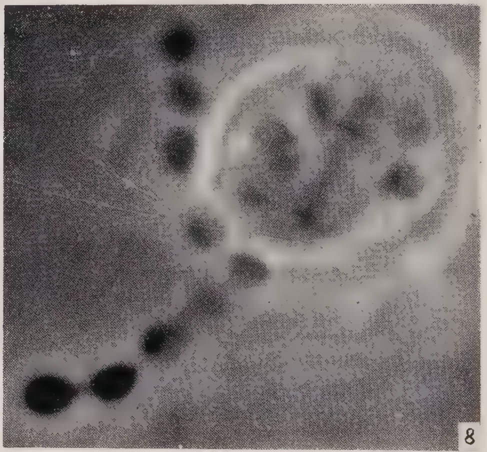

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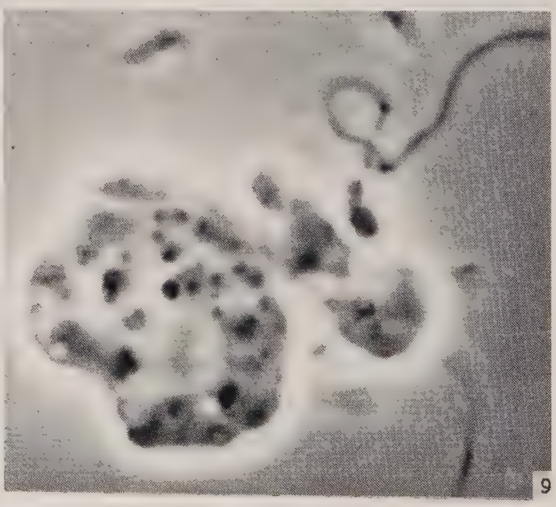

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S. L. CHANG—TRANSFORMATION OF *NAEGLERIA GRUBERI*. PLATE 2 within the survival period and of cysts up to about 1 week increased the potentiality of flagellate transformation, probably due to dehydration during freezing.

Observations on the prolonging effect of low temperatures and on the shortening effect of high temperatures of the flagellate stage, support the statement that flagellates are non-feeding forms and their ceaseless motion results in a rapid exhaustion of stored food, stimulating return to the amoeboid form. The higher the temperature, the more rapid is the movement and the more rapid is the exhaustion of the stored food.

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EXPLANATION OF PLATES

Plate 1

Phase-contrast micrographs of flagellate forms of *N. gruberi*.

Fig. 1. A trophozoite during the early stage of flagellate transformation.

Fig. 2. A flagellate form showing basal body at the base of each pair of flagella. Notice the change to amoeboid shape.

Figs. 3, 4. Late flagellate forms showing the alternating light and dark bands in flagella. Notice the change to amoeboid shape.

Plate 2

Figs. 5–7. Flagellate forms immobilized by $AgNO_3$. Notice the basal body in fig. 5, cytostome-like appearance in fig. 6, two pairs of flagella in fig. 7, and anteriorly located nucleus in all.

Figs. 8, 9. Flagellate forms reverting to amoebae. Notice the beaded appearance of flagella in fig. 8 and the separation of a small piece of cytoplasm and flagella from the body in fig. 9.

(*Received* 30 *September* 1957)

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Isolation of Lac$^+$ Mutants from a Lac$^-$ Strain of *Escherichia coli*, by the Replica Plating Technique

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SUMMARY: Lactose-fermenting (lac$^+$) mutants were isolated from a non-lactose-fermenting (lac$^-$) strain of *Escherichia coli*, strain O 110, by the replica plating technique. When high dilutions of a 24 hr. broth culture of the strain were plated on Endo agar, to give discrete colonies, secondary lac$^+$ colonies began to appear in the initially lac$^-$ colonies after 3 days' incubation; after 10 days, over 50 % of colonies contained secondary lac$^+$ colonies. Twenty serial subcultures were made in broth containing lactose, each subculture being incubated for only 6 hr. to prevent selection of lac$^+$ mutants from exhaustion of other nutrients while exposing the bacteria to lactose during many generations. When heavy inocula from the final subcultures were plated on Endo agar, no more lac$^+$ colonies appeared than from control platings of broth cultures which had not been exposed to lactose. An R variant of the lac$^-$ strain of *E. coli*, strain O 110, was found to assimilate lactose to a considerable extent without fermentation; the original S form possessed this capacity only to an insignificant extent.

The question whether lac$^+$ variants of *Bacterium coli mutabile* arise by spontaneous mutation or by adaptation is not yet solved definitively. Some authors (Monod, 1952; Ryan, 1952) maintain that mutation is a necessary preliminary to enzymic adaptation. Other authors (Dean & Hinshelwood, 1954) have concluded that the lac$^+$ variants originate by an adaptive process, and that the necessary key structures enabling the bacterium to adapt do not have to arise *de novo* in the course of the immediately observable history of the bacterium, but might have arisen at an earlier stage in the evolution of the strain.

The purpose of this study was to determine by the replica plating technique (Lederberg & Lederberg, 1952) whether lac$^+$ variants may originate in broth cultures of a lac$^-$ strain of *Escherichia coli*, strain O 110, regardless of contact with lactose.

MATERIALS AND METHODS

In the experiments described below a lac$^-$ strain of *Escherichia coli*, strain O 110, supplied by Dr F. Kauffmann (Statens Seruminstitut, Copenhagen), infusion broth, lactose broth (infusion broth containing 1% lactose), 3 % infusion agar, lactose + ammonium sulphate agar ($Na_2HPO_4 . 12H_2O$, 8 g; KH_2PO_4, 1·5 g; $(NH_4)_2SO_4$, 0·6 g; $MgSO_4 . 7H_2O$, 0·05 g; distilled water, 800 ml.; 5 % lactose, 100 ml.), and Difco Endo agar were used.

We used a modified replica plating technique, using—instead of velvet or velveteen—rubber disks with cylindrical excrescencies (such as used for covering table-tennis bats), glued to round glass plates. For the details of the technique see Banič (1956, 1958).

The broth culture of the lac$^-$ strain of *Escherichia coli*, strain O 110, was initiated at the beginning of both sets of replica experiments by plating a stock strain in S (smooth) form on infusion agar and subculturing a single colony to broth.

The initial growth on the infusion agar to be replicated was obtained by spreading 0·05 ml. of 18 hr. undiluted broth culture on infusion agar, and incubating the plate 5 hr. at 37°. For each subsequent selection stage the chosen broth cultures were more and more diluted (see below). In each selection stage replicas were transferred to one infusion agar plate and two lactose + ammonium sulphate agar or two Endo agar plates.

At the beginning, infusion agar and lactose + ammonium sulphate agar were used for the replica technique experiments, on the erroneous assumption that only lac$^+$ mutants will grow on this latter medium. Later, lactose + ammonium sulphate agar was replaced by Endo agar.

RESULTS

In the experiments with lactose + ammonium sulphate agar it was possible to isolate in pure culture, by the replica technique (after six selection stages), a strain which was able to form colonies *c.* 1 mm. diam. on lactose + ammonium sulphate agar after 2 days' incubation. The strain was plated on Endo agar to check whether it was indeed a lactose fermenter. To our great surprise it was found not to be a lactose fermenter but only a R (rough) form.

In the replica experiments with Endo agar, after the first selection stage, only 3 lac$^+$ colonies were noted at congruent sites on both Endo agar plates after 3 days' incubation (Pl. 1, figs. 1*a*, 1*b*, 1*c*). All three colonies were localized on the infusion agar master plate. These localized areas of growth were separately inoculated into broth. After 18 hr. incubation the broth cultures were diluted 10^{-1}, 0·05 ml. inoculated on to Endo agar plates, and the plates incubated. In the meantime the undiluted broth cultures were held in the refrigerator. After 3 days' incubation the lac$^+$ colonies on the Endo plates were counted. The broth culture found to contain the greatest number of lac$^+$ mutants was then diluted 10^{-1} and 0·05 ml. used to initiate growth on the infusion agar plate to be replicated in the second stage of indirect selection. After 5 hr. growth, replicas were then transferred to one infusion agar plate and two Endo agar plates. In this second stage 12 lac$^+$ colonies grew at congruent sites on both Endo agar plates after 3 days' incubation. Ten of these colonies were localized on the infusion agar plate, inoculated separately into broths and incubated 18 hr. As before, the broth culture yielding the greatest number of lac$^+$ mutants Endo agar in a pilot experiment, was chosen to prepare the master plate for the third selection stage.

In this way it was possible to isolate lac$^+$ mutants in pure culture after six stages of indirect selection.

The lac$^+$ strain thus obtained continued to breed true after 30 subcultures in infusion broth.

Three additional experiments were then carried out.

Experiment 1. 0·05 ml. of an undiluted 18 hr. broth culture of the original lac$^-$ strain of *Escherichia coli*, strain O 110, grown from a small inoculum (about 200 bacteria), was spread over the surface of each of 3 Endo agar plates. The plates were incubated 7 days and examined daily. After 1 day no lac$^+$ colony was observed on any plate. After 2 days some lac$^+$ colonies developed on all 3 plates; 24 hr. later the size of these colonies had increased. Plate 1, fig. 2, shows one of the 3 plates after 3 days' incubation. On this plate 4 lac$^+$ colonies had grown. After further incubation more lac$^+$ colonies developed.

Experiment 2. 0·05 ml. of an 10^{-5} dilution of a broth culture of the original lac$^-$ strain of *Escherichia coli*, strain O 110, initiated by a small inoculum, was spread over the surface of each of 3 Endo agar plates. The plates were incubated 10 days and examined daily. After 3 days', in 1–3 colonies on every plate a single secondary lac$^+$ colony was noticed. Plate 1, fig. 3, shows one of the plates, on which one secondary lac$^+$ colony is easily discernible. Plate 1, fig. 4, shows a colony from another plate containing (in the centre) a small secondary lac$^+$ colony at the very beginning of development. After further incubation of the plates, secondary colonies developed in more than 50% of the colonies. In the majority of these colonies only one secondary colony, but in a few colonies two to three secondary colonies, were observed.

Experiment 3. 10 ml. infusion broth containing 1% lactose (lactose broth) was inoculated by 0·05 ml. of a 10^{-5} dilution of a 24 hr. broth culture of the original lac$^-$ strain of *Escherichia coli*, strain O 110. After 6 hr. incubation 0·1 ml. of the well mixed culture was transferred to a new lactose broth. Altogether 20 subcultures were made, and every subculture was incubated only 6 hr. During the night the cultures were held at 4° to prevent the multiplication of the bacteria. After 20 subcultures in lactose broth, 0·05 ml. of the final well-mixed undiluted broth culture was spread on each of 3 Endo agar plates. The plates were examined after 3 days' incubation. On each of the plates only a few lac$^+$ colonies developed, as in Expt. 1.

DISCUSSION

The results of the replica plating experiments clearly demonstrate that lac$^+$ mutants arise in cultures of the lac$^-$ strain of *Escherichia coli*, strain O 110.

It was inferred from Expt. 1 that the mutation rate to lac$^+$ is extremely low in our lac$^-$ strain of *Escherichia coli*, strain O 110. Lac$^+$ colonies on Endo agar after 2 days of incubation probably develop from the mutants already present in the inoculum. Lac$^+$ colonies arising only after prolonged incubation probably develop from new mutants appearing during the growth of the culture on Endo agar.

Expt. 2 was performed because of the statement of Dean & Hinshelwood (1954) that almost every colony may become lac$^+$ when a small number of cells is plated on lactose + ammonium sulphate agar. Dean & Hinshelwood have interpreted the results of their experiments as indicating a change from lac$^-$ to lac$^+$ in the bulk, and not in a minute proportion, of the cells. Expt. 2, however, strongly suggests that this change is confined to a few bacteria.

The secondary lac$^+$ colonies within the initial lac$^-$ colonies doubtless develop from one bacterium, and the fact that the lac$^-$ colonies contain only 0–3 secondary colonies make it clear that the change from lac$^-$ to lac$^+$ occurs only in a minute proportion of the lac$^-$ bacteria. In the cited experiments of Dean & Hinshelwood, in our opinion the colonies became lac$^+$ because of the development of secondary lac$^+$ colonies which were difficult to observe since lactose + ammonium sulphate agar without indicator was used.

The author failed to isolate lac$^+$ mutants in the early experiments with lactose + ammonium sulphate agar. The single strain isolated was established as a R form of *Escherichia coli*, strain O 110, and nothing else. From this it was concluded that the R forms of lac$^-$ strains of *E. coli*, strain O 110, are able, without fermentation, to utilize lactose to a considerable extent as a source of energy. As no growth was observed on ammonium sulphate agar without lactose, impurities in the lactose preparation might have been responsible for the growth, but the growth in the presence of lactose was too abundant to allow such a possibility. Moreover, it was observed that our lac$^-$ strain of *E. coli*, strain O 110, in the *S* form was also able to grow to a small extent on lactose + ammonium sulphate agar, but not on the ammonium sulphate agar without lactose. The evidence therefore suggests that the lac$^-$ strain of *E. coli*, strain O 110, can utilize lactose without fermentation, to a very small extent in the S form and to a rather considerable extent in the R form.

Admittedly, the possibility of isolating lac$^+$ mutants by the replica plating technique does not prove *per se* that lac$^+$ variants originate only by spontaneous mutation. The result of Expt. 3, however, do lead to this conclusion. In this experiment, the opportunity for adaptation (growing in the presence of lactose), but not for selection of lac$^+$ mutants, existed since a lactose broth culture, after an incubation period of 6 hr., still contains enough nutrient substances other than lactose, so that the lac$^+$ mutants do not yet have a selection advantage. It could be objected that the experiment would have been better performed on a lactose + ammonium sulphate medium on which the bacteria were 'compelled' to adapt themselves because their sole source of carbon was lactose. Since lactose + ammonium sulphate medium acts selectively on the lac$^+$ mutants, however, such an experiment is out of the question.

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EXPLANATION OF PLATE

Fig. 1*a*. Replica to plain agar plate.

Fig. 1*b*. Replica to the first Endo agar plate. Note 5 lac$^+$ colonies (marked with arrows).

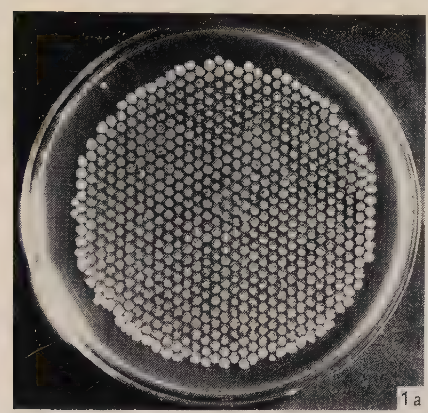
Fig. 1*c*. Replica to the second Endo agar plate. Note 3 lac$^+$ colonies at the congruent sites as in fig. 1*b*.

Fig. 2. Diffuse growth of a lac$^-$ strain of *Escherichia coli* on Endo agar plate. Note 4 lac$^+$ colonies. Incubation period 3 days.

Fig. 3. Colonies of a lac$^-$ strain of *E. coli* on Endo agar plate. Note in one colony a secondary lac$^+$ colony. Incubation period 3 days.

Fig. 4. Colony of a lac$^-$ strain of *E. coli* on Endo agar. Note in the centre of the colony a small secondary lac$^+$ colony. Incubation period 3 days.

(*Received* 16 *October* 1957)



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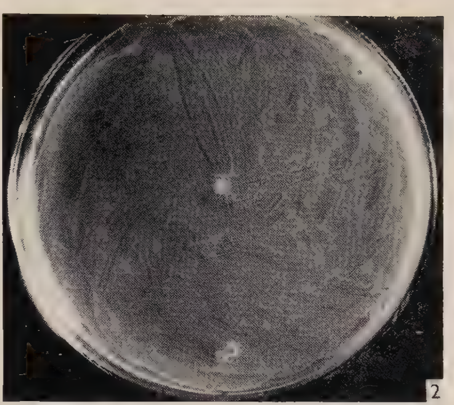

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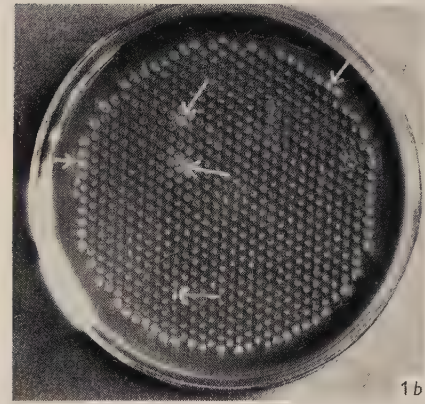

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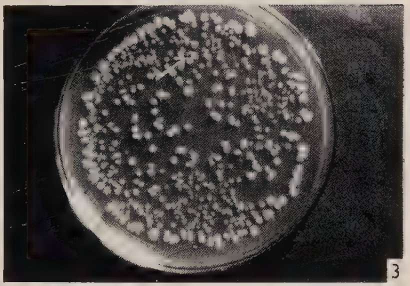

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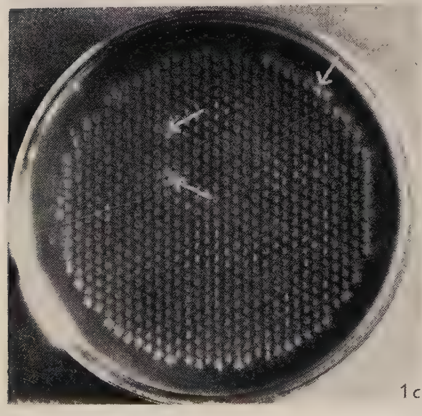

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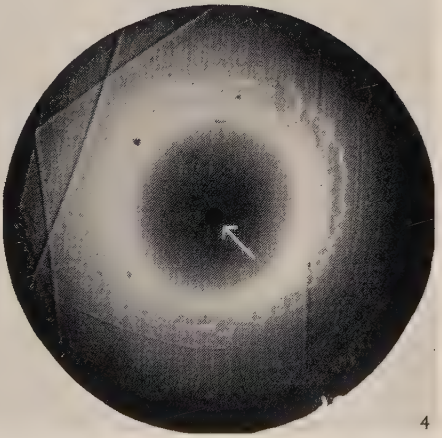

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S. BANIĆ—ISOLATION OF COLI MUTANTS. PLATE 1

(*Facing p.* 590)

Bradley, S. G. (1958). *J. gen. Microbiol.* 18, 591–596

Mechanism of 'Vegetative Hybridization' in Streptomyces

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SUMMARY: A population of *Streptomyces griseus*, strain W107, exposed for several growth cycles to a sterile culture filtrate of *S. griseus*, strain M142, acquired several genetic characteristics similar to those of strain M142. The changes observed were: (1) streptomycin sensitivity to resistance; (2) bacteriophage V_1 sensitivity to resistance; (3) absence to presence of soluble pigment; (4) presence to absence of pigment in the vegetative mycelium. The filtrate contained streptomycin and a temperate bacteriophage. The low concentration of streptomycin did not grossly inhibit the growth of strain W107 but streptomycin-resistant mutants were selected. Resistance to the temperate phage frequently conferred resistance to bacteriophage V_1. The observed morphological changes were coupled with bacteriophage- and streptomycin-susceptibility. Vegetative hybridization was the result of selection of mutants rather than gene transfer.

The streptomycetes are filamentous bacteria which resemble the true moulds in many respects. Hyphal fusion with resulting heterokaryosis has been demonstrated for several true moulds (Beadle & Coonradt, 1944) and for different species of *Streptomyces* (Bradley, 1957*a*). Specialized sexual structures have not been found in the streptomycetes. Sexuality in Streptomyces (Sermonti & Spada-Sermonti, 1956) probably resembles either parasexuality in Aspergillus or sexuality in *Escherichia coli* (Lederberg, 1952).

As is the case among true bacteria, many streptomycetes are lysogenic (Bradley, 1957*b*; Welsch, 1956). Temperate streptomyces-phages might serve as vectors for the transfer of genetic material. Alteration of the genetic composition of Streptomyces populations by extracts which might contain bacteriophage has been reported (Horvath, Marton & Oroszlan, 1954). Several growth cycles in the presence of the extract were required before phenotypic shifts were detected. The characteristics affected were colonial morphology, pigmentation and antibiotic production. The mechanism responsible for this 'vegetative hybridization' was not amply studied. Similar changes in populations of *Streptomyces griseus* treated with a culture filtrate have now been observed. These phenotypic shifts might result from (1) phage-mediated transduction; (2) deoxyribonucleic acid-mediated transformation; (3) selection for particular phenotypes. This paper presents evidence that the culture filtrate used contained selective agents. Some of these have now been identified.

METHODS

Mutant substrains of *Steptomyces griseus* were used throughout this investigation. The genetic markers employed were growth-factor requirements, antibiotic sensitivity, bacteriophage sensitivity and morphology. The characteristics of the mutant and ancestral substrains are shown in Table 1. The mutant substrains were obtained after ultraviolet irradiation or by direct selection using methods previously described (Bradley & Lederberg, 1956).

Table 1. *Principal stocks of* Streptomyces griseus *used and their ancestors*

Strain	Derived from	Genotype*
W 86	Nature	$V_1^s\ V_2^r\ V_3^s\ B^r\ S^s\ Ad^+\ \ Ar^+\ \ P_s^-\ P_v^+$
W 107	W 86	$V_1^s\ V_2^r\ V_3^s\ B^r\ S^s\ Ad^+\ (Ar^-)\ P_s^-\ P_v^+$
W 104	Nature	$V_1^s\ V_2^s\ V_3^r\ B^s\ S^s\ Ad^+\ \ Ar^+\ \ P_s^+\ P_v^-$
M 142	W 104	$\underline{V_1^r}\ V_2^s\ V_3^r\ B^s\ \underline{S^r}\ (Ad^-)\ Ar^+\ P_s^+\ P_v^-$

* V^s or V^r denotes sensitivity or resistance to bacteriophage. V_1, V_2 and V_3 represent bacteriophages WSP-3, WSP-2 and MSP-2 respectively. B^s or B^r denotes sensitivity or resistance to 2500 units bacitracin/l. S^s or S^r denotes sensitivity or resistance to 25 mg. dihydrostreptomycin sulphate/l. Ad^+ or Ad^- and Ar^+ or Ar^- denote independence or dependence for exogenous adenine or arginine 20 mg/L. P_s^+ or P_s^- and P_v^+ or P_v^- denote presence or absence of soluble pigment and pigment in the vegetative mycelium respectively.

( ), genetic markers obtained after ultraviolet irradiation.

——, genetic markers obtained by direct selection.

The basal medium was composed of: glucose, 20 g; KNO_3, 2 g.; K_2HPO_4, 2 g.; $MgSO_4.7H_2O$, 0·5 g.; $CaCO_3$, 0·25 g.; water, 1 l. For complete medium, 0·1 % (w/v) Difco yeast extract was added. The medium was solidified with 1·5 % (w/v) agar. Glucose and salts solutions were autoclaved separately at double strength.

The culture filtrate was prepared as follows. Complete medium was inoculated with *Streptomyces griseus*, strain M 142, to yield 10^6–10^7 spores/ml. and incubated at 30° on a rotary shaker. The culture underwent spontaneous lysis after 4 days and the culture was then filtered through coarse filter-paper to remove floating debris and remaining mycelium. The culture filtrate was further clarified by centrifugation and rendered bacteriologically sterile by filtration.

RESULTS

Streptomyces griseus, strain W 107, was grown in complete medium to which 5 % (v/v) strain M 142 filtrate had been added. After several serial subcultures exposed to the filtrate, non-parental types appeared, with respect to the streptomycin and bacteriophage V_1 characters. The strain M 142 culture filtrate did not affect the bacteriophage V_3 and arginine characters (Table 2). These data, taken from a typical experiment, have only slight quantitative significance because 'colony forming unit' is not a distinct entity. Colony forming unit refers to a hyphal fragment of any size which produces a single discrete colony. The number of subcultures required to establish non-parental types varied considerably as between different experiments. Rarely non-parental types arose as early as the second subculture and in one experiment, non-parental types did not become established until the twelfth subculture.

Treated strain W 107, selected for one non-parental character, frequently differed for other characters as well (Table 3). Growth-factor requirements, bacitracin sensitivity and bacteriophage V_3 sensitivity were not altered in the selected streptomycin- or bacteriophage V_1-resistant substrains. Other factors were affected to different degrees, exceeding the spontaneous mutation rate, which was less than 10^{-3}.

Table 2. *Selection for non-parental phenotypes of* Streptomyces griseus *strain W*107

No. subcultures in strain M142 filtrate	Characteristics selected S^r	 V_1^r	 V_3^r	 Ar^+
	Colonies/10^7 colony-forming units			
0	0	20	7	0
1	0	31	4	0
2	0	18	4	0
3	0	59	9	0
4	27	132	11	0
5	897	1021	3	0
6	$3{\cdot}3\times10^6$	$1{\cdot}7\times10^6$	0	0

Table 3. *Characteristics of non-parental isolates of* Streptomyces griseus *strain W*107

Strain W107 was grown for 9 subcultures in the presence of 5% (v/v) strain M142 or W104 culture-filtrates.

Filtrate used	Character selected	Character V_1	 V_2	 V_3	 B	 S	 P_s	 P_v	 Ad	 Ar
		% different from strain W107								
M142	Control, no selection	13	4	<0·1	<0·1	35	2	9	<0·1	<0·1
M142	S^r	27	<0·1	<0·1	<0·1	100	9	14	<0·1	<0·1
M142	V_1^r	100	38	<0·1	<0·1	13	0·6	15	<0·1	<0·1
W104	Control, no selection	24	7	0·5	<0·1	<0·1	<0·1	2	<0·1	<·01

A filtrate of strain M142, heated at 65° for 30 min., was added to strain W107 cultures. No strain W107 organism, resistant to bacteriophage V_1, was found during any of the serial subcultures. The development of streptomycin resistance was not affected. This indicated that at least two distinct agents were involved.

Strain M142 is lysogenic (Bradley, 1957*c*). Antisera against the temperate phage L3 was added to the strain W107 cultures, in addition to the M142 culture-filtrate. No bacteriophage V_1-resistant organisms were isolated during the serial subcultures. As with heat-treated filtrates, antisera did not affect the development of streptomycin resistance. The temperate phage L3 was responsible for the increased proportion of V_1^r cells.

The ancestor of strain M142, i.e. W104, is streptomycin sensitive, phage V_1 sensitive and lysogenic for the temperate phage L3. Filtrates of strain W104 added to growing cultures of W107 produced the change from phage V_1 sensitivity to resistance as effectively as filtrates of strain M142. However, the W104 filtrate did not affect the streptomycin character (Table 3). As before, the number of subcultures required to attain corresponding yields of the non-parental types varied considerably as between different experiments.

Table 4. *Factors affecting the activity of* Streptomyces griseus *strain* M142 *filtrates*

Treatment	Composition of W107 population after 8 subcultures			
	V_1^r	V_3^r	S^r	Ar^+
	Colonies/10^7 colony-forming units			
65° for 30 min.	83	0	$7{\cdot}1 \times 10^6$	0
Anti-L3 serum	0	0	$4{\cdot}9 \times 10^6$	0
Activated charcoal	$9{\cdot}6 \times 10^5$	0	0	0
pH 2 eluate from charcoal	61	0	$1{\cdot}0 \times 10^7$	0
Control, no treatment	$1{\cdot}4 \times 10^6$	0	$3{\cdot}2 \times 10^6$	0

Bacteriophages L3 and V_1 are demonstrably different. However, resistance to L3 might concurrently confer resistance to V_1. This was shown to occur. Of 63 mutants of strain W107 selected for resistance to L3, five were also resistant to V_1. Moreover, two were resistant to V_3 and one lacked pigment in the vegetative mycelium. Resistance to the temperate phage L3 might result by selection of a mutant or by lysogenization. If W107 were lysogenized, free phage should be demonstrable in culture autolysates. Bacteriologically sterile filtrates of phage V_1-resistant derivatives of strain W107 were plated with sensitive indicator strains. No evidence of lysogenization was found. The temperate phage L3 was irregularly found in lysates of strain M142. Therefore the temperate phage L3 was the selective agent responsible for certain changes in morphology and bacteriophage susceptibility.

Activated charcoal removed the factor in the strain M142 filtrate (pH 7·0) responsible for the change from streptomycin sensitivity to resistance. An eluate made by extracting the activated charcoal with acidified water (pH 2) was able to alter streptomycin susceptibility. The eluate inhibited completely the growth of strain W107 unless the eluate was adequately diluted. This indicated that strain M142 might produce an antibiotic. The filtrate inhibited streptomycin-sensitive but not streptomycin-resistant strains of *Escherichia coli*. Comparing the inhibitory effect of the culture filtrate and dihydrostreptomycin sulphate standards, it was found that strain M142 produced antibiotic equivalent to 30–40 mg. streptomycin/l.

The low concentration of streptomycin added to the experimental flasks was not sufficient to alter grossly the growth of strain W107. However, W107 grown for 10 subcultures in 2 mg. dihydrostreptomycin sulphate/l. became resistant to 25 mg./l. A few of these colonies on complete agar produced soluble pigment (7 of 1291) and several lacked pigment in the vegetative mycelium (122 of 1291).

DISCUSSION

Culture filtrates of *Streptomyces griseus*, strain M142, brought about certain genetic changes in *S. griseus*, strain W107. Several growth cycles in the filtrate were required before these changes became detectable. Selective agents produced by strain M142 were responsible. Two of the agents, temperate bacteriophage L3 and streptomycin, were isolated from the M142 filtrates.

To demonstrate conclusively that the genesis of resistance to bacteriophage V_1 was the result of selection of mutants resistant to temperate bacteriophage L3, it was necessary to rule out DNA-mediated transformation, phage-mediated transduction and cross-resistance conferred by lysogenization. Antiserum against temperate phage L3 prevented the development of V_1 resistance. Temperate phage L3 was essential, therefore DNA-mediated transformation was not the mechanism involved. Moreover, L3 grown on a V_1-sensitive donor promoted the development of V_1 resistance as effectively as phage L3 grown on strain M142. This was inconsistent with both DNA-mediated transformation and phage-mediated transduction. Temperate phage L3 could not be isolated from autolysates of the V_1-resistant derivatives of strain W107. This indicated that lysogenization had not occurred.

Quantitative comparisons among the streptomycetes were difficult because 'cell number' could not be accurately enumerated. Moreover, genetically-altered nuclei might be masked by normal nuclei in the same cytoplasm. The alterations resulting from dihydrostreptomycin sulphate and phage L3 did not precisely duplicate the effect of the culture filtrate. It is possible that other selective agents were involved. Metabolic products such as alanine and kinetin select for particular phenotypes of Brucella (Mika, Braun, Ciaccio & Goodlow, 1954; Braun, Firshein & Whallon, 1957). Horvath *et al.* (1954) interpreted similar data as transduction or transformation. Selection of mutants was ruled out because the observed changes grossly occurred during a single subculture, after several subcultures in presence of the cell extract. Gross populational changes cannot be used as an accurate test of selection. The choice of genetic markers is important for interpretation of experimental data. In Streptomyces, antibiotic resistance may be coupled with antibiotic synthesis. Cross-reaction among streptomyces-phages and the relatively high mutation rate from phage sensitivity to resistance makes this genetic marker difficult to score. Morphological expressions seemed to be related to antibiotic and bacteriophage sensitivity. Growth-factor requirements have not been found, in this study, to be coupled with morphology or susceptibility. Growth-factor requirements are invaluable in genetic studies of the Streptomyces.

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The Phosphorus-containing Compounds of Gram-positive and Gram-negative Organisms in Relation to the Gram Staining Reaction

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SUMMARY: The phosphorus-containing compounds of certain Gram-positive and Gram-negative organisms were estimated by a modification of the Schmidt & Thannhauser (1945) procedure. Another method (Mitchell & Moyle, 1950, 1951*a*, *b*, 1954) in which pentosenucleic acid (PNA) was estimated from the optical absorption of the 'PNA fraction' at 260 mμ. was found to be inaccurate, because of the presence of ultraviolet-absorbing impurities. A more accurate method for estimating PNA has now been developed, in which the nucleotides are separated from impurities by paper electrophoresis. The results thus obtained show that non-nucleotide phosphorus-containing compounds are present in the 'PNA fraction' of both Gram-positive and Gram-negative organisms. There is, therefore, no evidence to connect these compounds with the Gram staining properties of Gram-positive micro-organisms (cf. Mitchell & Moyle, 1950, 1951*a*, *b*, 1954).

It has been shown that magnesium ribonucleate, in association with certain proteins and possibly polysaccharides, is the complex responsible for the retention of the Gram stain in Gram-positive micro-organisms (Henry & Stacey, 1943, 1946; Henry, Stacey & Teece, 1945; Jones, Muggleton & Stacey, 1950). These conclusions, which have been largely confirmed by other workers (Bartholomew & Umbreit, 1944) have since been questioned by Mitchell & Moyle (1950, 1951*a*, *b*, 1954) who claimed that Gram-positive organisms contain a particular phosphoric ester fraction (XSP) which is absent from Gram-negative species and that this 'XSP factor' is responsible for the retention of the stain in the Gram reaction. In view of these claims, it was decided to re-investigate the problem and in particular to analyse various micro-organisms for their XSP content.

METHODS

Organsisms used and methods of cultivation. The following organisms were used: (*a*) Gram-positive; baker's yeast (Distillers Co.), *Bacillus subtilis*, *Clostridium welchii*, *Sarcina lutea*, *Streptococcus faecalis* (NCTC, 370);. (*b*) Gram-negative; *Aerobacter aerogenes* (NCTC 8172), *A. cloacae* (NCTC 8813), *Escherichia coli* and *Klebsiella pneumoniae* (NCTC 3279). They were grown in a medium containing (%, w/v): 0·5, Lab Lemco; 1, Oxoid bacteriological peptone; 1, glucose; 0·5, sodium chloride. The solution of Lab Lemco, peptone and sodium chloride was adjusted to pH 9, boiled for 5–10 min. and the resulting precipitate filtered off. The filtrate was adjusted to pH 7·5 and the solution autoclaved at 10 lb./sq.in. for 15 min. Sterile glucose solution was then added aseptically. The complete medium was inoculated with an

18 hr. culture of the required organism. The cultures of *Aerobacter aerogenes*, *A. cloacae*, *Bacillus subtilis* and *Sarcina lutea* were aerated during growth. After incubation at 37° for 18 hr. (except for *S. lutea* which was grown for 48 hr.) the organisms were centrifuged down, washed with 0·85% (w/v) sodium chloride and freeze-dried. The *Klebsiella pneumoniae* cultures were autoclaved at 15 lb./sq.in. for 10 min. before centrifugation.

Determination of the phosphorus-containing compounds. The determination of phosphorus-containing compounds was initially carried out by Mitchell & Moyle's (1951*a*, *b*, 1954) method (summarized in Fig. 1), which was an adaptation of the Schmidt & Thannhauser (1945) procedure, thus giving an estimate of the following fractions: acid-soluble inorganic phosphorus (AI); acid-soluble organic phosphorus (AO); lipid phosphorus (L); pentosenucleic acid phosphorus (PNAP); deoxypentose nucleic acid phosphorus (DNAP); phosphoprotein phosphorus; XSP phosphorus. Phosphorus was determined as described by Jones, Lee & Peacocke (1951). After extraction of the lipids the organisms were dried *in vacuo* over silica gel instead of over phosphorus pentoxide in order to eliminate any possibility of contamination with the latter. The XSP phosphorus was obtained by subtracting from the total phosphorus of the 'PNA fraction' the phosphoprotein phosphorus (estimated as the inorganic phosphorus of the PNA fraction) and the PNA phosphorus, the latter being calculated from the optical extinction of the solution at 260 mμ. For this purpose Mitchell & Moyle assumed that the absorption of other components at 260 mμ. was negligible and that the ϵ_p value of all the pentose nucleic acids was 10,000 under the conditions of the estimation. ($\epsilon_p = 30{\cdot}98E/cl$, where E = extinction of the solution; c = concentration of phosphorus in g./l.; l = thickness of absorbing layer in cm.; Chargaff & Zamenhof, 1948.)

It became obvious, however, that this method of estimating PNA was inaccurate, both due to the fact that absorption at 260 mμ. of components other than nucleotides was far from negligible and that the ϵ_p values of PNA vary according to the source and are not equal to 10,000. The PNA was, therefore, subsequently determined by separation of the nucleotides of the PNA fraction by paper electrophoresis on Whatman No. 3 paper at pH 3·5 for 8·5 hr. at 1400 volts by Crosbie, Smellie & Davidson's (1953) method as previously described (Foster, Jones & Rizvi, 1956). The nucleotides were eluted from the paper with phosphate buffer (pH 7) and estimated spectrophotometrically. With pure yeast PNA 95% recovery was obtained. When applied to the PNA fractions of the micro-organisms, good separation of the nucleotides was not obtained, however. Since this was thought to be due to the presence of salts, N-KOH was used to digest the organisms instead of N-NaOH, and perchloric acid to precipitate DNA at pH 1 instead of trichloroacetic and hydrochloric acids. After separating the DNA, protein, and potassium perchlorate precipitates by centrifugation, the supernatant liquid was adjusted to pH 4 and freeze-dried. The nucleotides were then extracted from the freeze-dried material with 60% (v/v) ethanol in water and applied to the paper. With pure yeast PNA, 95% recovery was obtained. This showed that there was a negligible loss of nucleotides on the potassium perchlorate precipitate (cf. Elson, Trent

& Chargaff, 1955). The non-interference of other cell constituents in the separation was established by estimating the nucleotide content of yeast PNA; in presence and in absence of a yeast extract. Satisfactory separation of the nucleotides was obtained and the recovery of PNA was again 95%. Later work showed that the analysis could be improved in the following respects. (1) It was not necessary to adjust the pH value of the solution of nucleotides from pH 1 to pH 4 in order to achieve separation. (2) Whatman No. 1 paper could be used instead of Whatman No. 3, thereby decreasing the ultraviolet absorption of the blanks and simplifying the elution of the nucleotides. (3) Freeze drying of the PNA fraction and subsequent extraction of the nucleotides with 60% (v/v) ethanol in water was not necessary if, after placing the ionophoretogram on the glass plates in the usual manner, it was left for 15 min. before passing the current. This allowed all the material to dissolve in the buffer and so prevented undissolved fragments of the PNA fraction from interfering in the separation of the nucleotides. (4) More rapid and complete extraction of the nucleotides from the paper was obtained by the use of 0·1 N-HCl at 37° for 18 hr. instead of phosphate buffer of pH 7. Degradation of the nucleotides was negligible and the recovery of PNA was in this case 97–100% of the theoretical. The XSP content was then determined using this more accurate value for the PNA phosphorus. The final procedure is shown in Fig. 1.

RESULTS

Distribution of phosphorus-containing compounds in micro-organisms

A number of Gram-positive and Gram-negative micro-organisms were analysed by the methods described above. The results (Table 1) show several marked differences from those obtained by Mitchell & Moyle. Using the method of these authors for calculating the XSP content, the distinction between Gram-positive and Gram-negative organisms was not nearly so marked as the earlier results (Mitchell & Moyle, 1954) suggested. Thus, although, in general, the Gram-positive organisms appeared to contain more XSP factor than did the Gram-negative species, one or two anomalies were found; *Klebsiella pneumoniae* appeared to have a higher XSP content than did baker's yeast and *Aerobacter aerogenes* had an appreciable 'negative XSP content'.

From the results obtained by using the electrophoretic method for the estimation of PNA, it was obvious that all the organisms had an appreciable XSP content and that the low values obtained for Gram-negative organisms by Mitchell & Moyle's method were due mainly to the presence of non-nucleotide, ultraviolet-absorbing material in the PNA fraction and, to a less extent, to the use of an inaccurate ϵ_p value for the estimation of PNA. The ϵ_p^{260} values for the PNA from various micro-organisms are shown in Table 2.

Non-nucleotide ultraviolet-absorbing components

The ultraviolet (u.v.) absorption spectra of the PNA fractions from all the organisms studied were not typical of pure ribonucleotides, the absorption at 220 mμ. being much higher than that at 260 mμ. (Figs. 2 and 3 show curves

Table 1. *Distribution of phosphorus-containing compounds in certain micro-organisms*

Code for fractions: L=Lipid phosphorus; AI = acid-soluble inorganic phosphorus; AO = acid soluble organic phosphorus; DNAP = DNA phosphorus; PNAP = PNA phosphorus; XSP = 'Excess phosphorus' in the PNA fraction.

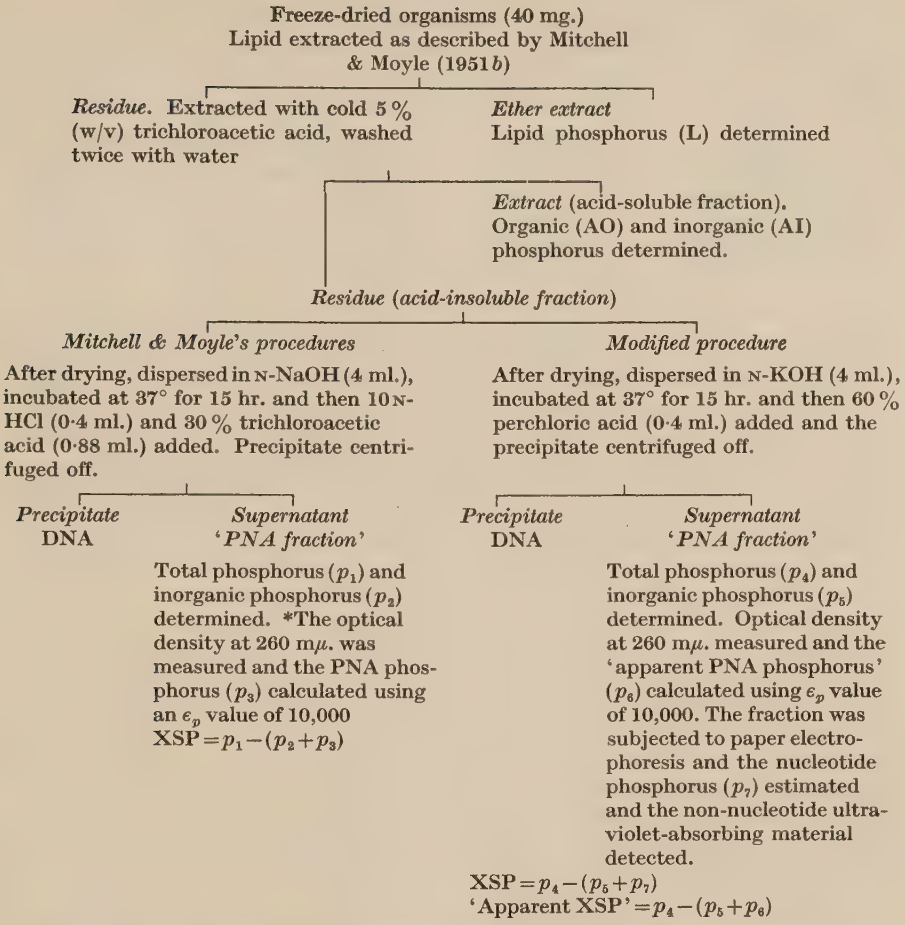
Methods of determination: (*a*) = determined by Mitchell & Moyle (1954); (*b*) = determined by Mitchell & Moyle's (1951*a*, *b*, 1954) method; (*c*) = determined by the present method.

		µmole phosphorus/g. freeze-dried organism. Fraction																						
		Total			L			AI			AO			DNAP			PNAP			XSP			XSP (% total P)	Non-nucleotide u.v.-absorbing material
		Method of determination																						
Organism	Gram reaction	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)	(*c*)	(% of PNA)*
Sarcina lutea	+	.	411	411	.	6·12	6·12	.	44·1	44·1	.	62·9	62·9	.	35.0	35·0	.	176	105	.	86·9	158	39·3	37·0
Bacillus subtilis	+	1157	734	734	16	7·30	7·30	70	98·6	98·6	238	54·0	54·0	71	58·0	58·0	291	292	188	471	22·4	338	45·8	31·5
Clostridium welchii	+	959	698	698	45	8·12	8·12	37	55·0	55·0	30	41·6	41·6	130	56·2	56·2	491	504	304	226	32·7	233	33·0	34·2
Streptococcus faecalis	+	1297 1047	685	685	26 29	29·8	29·8	146 200	62·2	62·2	266 129	87·0	87·0	168 111	65·4	65·4	459 417	406	278	232 161	34·5	162	23·6	28·6
Baker's yeast	+	352	328	328	9·4	24·0	24·0	47	71·4	71·4	45	19·3	19·3	22	20·6	20·6	169	169	130	60	24·3	63·6	19·3	19·0
Klebsiella pneumoniae	–	.	450	450	.	40·0	40·0	.	8·0	8·0	.	5·8	5·8	.	38·0	38·0	.	328	207	.	31·0	152	34·6	28·2
Aerobacter aerogenes	–	594	459	459	62	47·7	47·7	50	20·7	20·7	46	70·1	70·1	.	26·8	26·8	407	317	204	4	–22·1	91·0	19·8	30·6
A. cloacae† †	–	.	601	601	.	67·0	67·0	.	57·0	57·0	.	59·0	59·0	.	41·0	41·0	.	366	210	.	12·0	167	26·8	37·4
‡		.	.	578	.	.	68·0	.	.	47·0	.	.	53·0	.	.	45·0	.	.	210	.	.	155		
Escherichia coli‡ †	–	664	543	543	63	45·3	45·3	26	13·1	13·1	42	49·0	49·0	180	30·0	30·0	345	410	223	8	– 4·7	182	31·8	43·0
‡		.	.	540	.	.	48·0	.	.	17·6	.	.	49·9	.	.	28·0	.	.	224	.	.	172		

* Assuming that this material has the same optical absorption at 260 mµ. as has PNA.

† DNA precipitated with trichloroacetic and hydrochloric acids.

‡ DNA precipitated with perchloric acid.



Freeze-dried organisms (40 mg.)
Lipid extracted as described by Mitchell
& Moyle (1951*b*)

Residue. Extracted with cold 5 % (w/v) trichloroacetic acid, washed twice with water

Ether extract
Lipid phosphorus (L) determined

Extract (acid-soluble fraction). Organic (AO) and inorganic (AI) phosphorus determined.

Residue (acid-insoluble fraction)

Mitchell & Moyle's procedures

After drying, dispersed in N-NaOH (4 ml.), incubated at 37° for 15 hr. and then 10 N-HCl (0·4 ml.) and 30 % trichloroacetic acid (0·88 ml.) added. Precipitate centrifuged off.

Precipitate
DNA

Supernatant
'*PNA fraction*'

Total phosphorus (p_1) and inorganic phosphorus (p_2) determined. *The optical density at 260 mμ. was measured and the PNA phosphorus (p_3) calculated using an ϵ_p value of 10,000
$XSP = p_1 - (p_2 + p_3)$

Modified procedure

After drying, dispersed in N-KOH (4 ml.), incubated at 37° for 15 hr. and then 60 % perchloric acid (0·4 ml.) added and the precipitate centrifuged off.

Precipitate
DNA

Supernatant
'*PNA fraction*'

Total phosphorus (p_4) and inorganic phosphorus (p_5) determined. Optical density at 260 mμ. measured and the 'apparent PNA phosphorus' (p_6) calculated using ϵ_p value of 10,000. The fraction was subjected to paper electrophoresis and the nucleotide phosphorus (p_7) estimated and the non-nucleotide ultra-violet-absorbing material detected.

$XSP = p_4 - (p_5 + p_7)$
'Apparent XSP' $= p_4 - (p_5 + p_6)$

* In the present investigation a difficulty was encountered in determining the optical absorption of the PNA fraction due to the use of trichloroacetic acid as a precipitant for the DNA. Trichloroacetic acid has an appreciable absorption at 260 mμ. for which it was difficult to correct due to the absorption of an unknown quantity of trichloroacetic acid on the DNA and protein precipitate. In order to eliminate this uncertainty the PNA fraction was extracted four times with ether before the optical density was determined. A blank was obtained by similarly extracting a solution of trichloroacetic acid and hydrochloric acid of the same strength was used to precipitate the DNA. There is no indication of how the blank was determined by Mitchell & Moyle.

Fig. 1. Procedures used for the determination of the phosphorus-containing compounds in micro-organisms.

for two of the organisms; the others were similar.) This indicated the presence of other u.v.-absorbing components in this fraction. These could be separated from the nucleotides by paper electrophoresis at pH 3·5, when the nucleotides migrated to the anode and the other components either remained on the starting line or migrated to the cathode (Pl. 1, fig. 1). These components were eluted, combined, and their u.v.-absorption spectra determined. The spectra (Figs. 2, 3) were similar for the components from all the organisms and showed no definite peak. This absorption contributed on the average about 30% to the total absorption of the PNA fraction at 260 mμ. (Table 1).

Table 2. *ϵ_p^{260} values at* pH 1 *of PNA from various micro-organisms*

Organism	ϵ_p^{260}	Organism	ϵ_p^{260}
Bacillus subtilis	10,600	*Aerobacter aerogenes*	10,800
Clostridium welchii	10,900	*A. cloacae*	10,900
Sarcina lutea	10,600	*Escherichia coli*	10,500
Streptococcus faecalis	10,450	*Klebsiella pneumoniae*	10,900
Baker's yeast	10,600		

An alternative method for the isolation of these components was to freeze dry the PNA fraction and extract the nucleotides with 60% (v/v) ethanol in water. Most of the non-nucleotide u.v.-absorbing material remained insoluble. This insoluble material gave a positive ninhydrin reaction and a negative reaction for carbohydrate, and therefore appeared to be mainly peptide in nature. This was confirmed by hydrolysing the PNA fraction of *Klebsiella pneumoniae* with 5N-HCl and separating the products by paper electrophoresis at pH 5·0. Upon spraying the paper with ninhydrin, spots corresponding to basic, neutral and acidic amino acids were obtained.

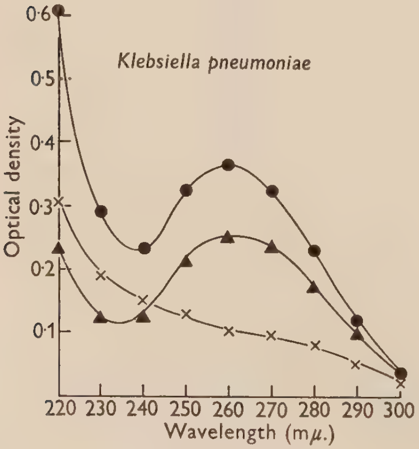


Fig. 2

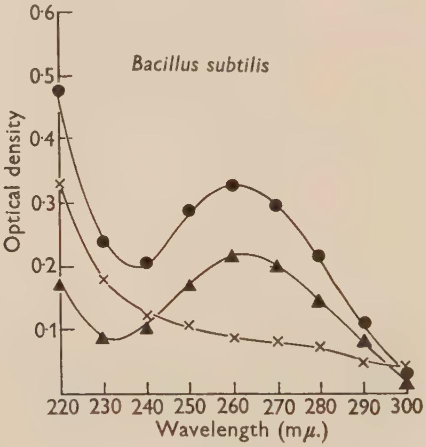


Fig. 3

Figs. 2, 3. Ultraviolet absorbing components of the PNA fractions of *Klebsiella pneumoniae* (Fig. 2) and of *Bacillus subtilis* (Fig. 3). ●—●, Total PNA fraction; ×—×, Non-nucleotide components; ▲—▲, nucleotides.

The 'XSP factor' of Klebsiella pneumoniae

Further evidence for the presence of non-nucleotide phosphorus-containing compounds (XSP) in *Klebsiella pneumoniae* was obtained by the use of radioactive phosphorus (^{32}P). The organisms were grown for 18 hr. at 37° in 500 ml. medium containing 3 millicuries (mc.) of ^{32}P as inorganic phosphate. The culture was then autoclaved, and the organisms centrifuged off, washed with 0·85% (w/v) sodium chloride and freeze dried. The PNA fraction was then prepared as usual and subjected to paper electrophoresis on Whatman No. 3MM paper at pH 3·5 for 17·75 hr at 900 V. The u.v.-absorbing compounds were detected in the usual way and the compounds containing ^{32}P detected by autoradiography. The results (Pl. 1, fig. 2) show that in addition to the four nucleotides there were present at least ten other phosphorus-containing compounds, some of which coincided with some of the non-nucleotide u.v.-absorbing components. Since this electrophoretogram was run for a considerably longer time than that shown in Pl. 1, fig. 1, some of the non-nucleotide u.v.-absorbing components had run off the end of the paper.

Some of the components of the XSP factor of *Klebsiella pneumoniae* were isolated and partly identified as follows (cf. Mitchell & Moyle, 1951*a*). The PNA fraction was prepared as previously described (without the use of ^{32}P) from 40 mg. freeze-dried organisms. This fraction was adjusted to pH 9 with ammonia, 95 % (v/v) ethanol in water added (1 vol.) and the mixture allowed to stand at room temperature for 2 days. The precipitate (non-nucleotide u.v.-absorbing material) was centrifuged off and to the supernatant liquid there was added M-$BaCl_2$ solution (0·5 ml.). The precipitate thus formed was centrifuged off, washed twice with 60 % (v/v) ethanol in water, extracted with 0·1 N-H_2SO_4 and the precipitate of barium sulphate centrifuged off. The supernatant liquid was adjusted to pH 5 with barium hydroxide solution and the barium sulphate precipitate centrifuged off.

In order to remove nucleotides from the supernatant liquid a charcoal slurry (5 mg. charcoal washed by the method of Lipkin, Talbert & Cohn, 1954, in 1 ml. distilled water) was added dropwise until all the nucleotides had been adsorbed. This was shown by the absence of a peak at 260 mμ. in the optical absorption curve of the solution after the removal of the charcoal. The resulting solution contained non-nucleotide u.v.-absorbing material, a small quantity of inorganic phosphate and 64 % of the XSP factor originally present in the PNA fraction. By a similar procedure, 63 % of the XSP factor of *Aerobacter cloacae* was obtained. The solution obtained from *Klebsiella pneumoniae* was freeze-dried and the components separated by paper chromatography by techniques described by Benson *et al.* (1951) and Hanes & Isherwood (1949).

Acid-washed Whatman No. 1 and No. 4 papers were used. The phosphates were detected by the use of the spray reagent described by Hanes & Isherwood (1949). After spraying, the papers were dried, heated at 80° for 10 min. and then exposed to u.v. radiation for 10 min. as recommended by Bandurski & Axelord (1951). After irradiation most of the organic phosphates appeared immediately as blue spots but some only appeared after some time (in one case 12–15 hr.).

When chromatograms were developed on Whatman No. 1 paper with *n*-propanol + water + ammonia (sp.gr. 0·880); (60:10:30), for 18 hr., five spots of R_F values 0, 0·11, 0·26, 0·38, and 0·67 were detected. When a mixture of *iso*propanol + water + ammonia (sp.gr. 0·880), (70:6:30), was used as the solvent on Whatman No. 1 paper after 25 hr. development, seven spots of R_F values 0, 0·065, 0·17, 0·26, 0·39, 0·54, and 0·69 were obtained, and on Whatman No. 4 paper after 9 hr. development, seven spots of R_F values, 0, 0·10, 0·33, 0·46, 0·62, 0·72, and 0·86 were detected. It appeared, therefore, that at least seven phosphorus-containing compounds were present. Table 3 shows that two of these corresponded in R_F values with α-glycerophosphate and inositol phosphate, respectively. The colour of the spot for inositol phosphate was lighter than that of the corresponding spot of the XSP factor. This may have been because in the latter case peptide material was present at the same position on the chromatograms.

Table 3. *R_F values of α-glycerophosphate, inositol phosphate and phosphate fractions of* Klebsiella pneumoniae

Solvent	Whatman Paper No.	Component or compound: α-Glycero-phosphate	Component of 'XSP' factor	Inositol phosphate	Component of 'XSP' factor
		R_F values			
n-Propanol + water + ammonia (sp.gr. 0·880); (60:10:30)	1	0·25	0·25	0·11	0·11
*iso*Propanol + water + ammonia (sp.gr. 0·880); (70:6:30)	1	0·54	0·54	0·39	0·39
	4	0·72	0·72	0·63	0·62

DISCUSSION

The results of this investigation show that pentose nucleic acid (PNA) cannot be estimated accurately in micro-organisms by the determination of either the optical absorption at 260 mμ. or the phosphorus content of the 'PNA fraction' of the Schmidt & Thannhauser (1945) procedure. This is due to the presence in this fraction of phosphorus-containing impurities and u.v.-absorbing impurities. An accurate estimation of PNA was obtained, however, by removing these impurities by paper electrophoresis and estimating the nucleotides spectrophotometrically. The use of this technique showed that both Gram-positive and Gram-negative organisms contained in their PNA fractions non-nucleotide, phosphorus-containing compounds (XSP factor). The results of Mitchell & Moyle (1950, 1954), from which it was concluded that only Gram-positive organisms contained this factor, were apparently due in part to their having ignored the presence of non-nucleotide u.v.-absorbing compounds.

The results obtained in this investigation for the XSP contents of the various micro-organisms were, where comparison was possible, much lower than those of Mitchell & Moyle when these authors' own method was used. It seems possible that this may have been due to the fact that in this work the organisms were grown in a Lemco + peptone medium at 37°, and that Mitchell & Moyle used a casein digest medium at 25°. In *Klebsiella pneumoniae*, an organism not analysed by Mitchell & Moyle, however, the XSP content was higher than any recorded by Mitchell & Moyle for a Gram-negative organism and higher than that found in this work in baker's yeast. When the present more accurate method of estimating PNA was used, however, the results for Gram-positive organisms were in fair agreement with those of Mitchell & Moyle but those for Gram-negative organisms were much higher.

The presence of 'XSP factor' in a Gram-negative organism has been confirmed both by the experiment with ^{32}P and by the isolation of non-nucleotide phosphorus-containing compounds from *Klebsiella pneumoniae.* Two of these have been tentatively identified as α-glycerophosphate and inositol phosphate, respectively.

The presence of non-nucleotide phosphorus-containing compounds in the PNA fraction of higher organisms has been reported by a number of workers. Davidson & Smellie (1952) found that this fraction from liver cells contained, in addition to the four nucleotides, at least six other phosphorus-containing compounds; and Logan, Mannell & Rossiter (1952) found that in nerve tissue, the non-nucleotide phosphorus-containing compounds accounted for more than 50% of the total phosphorus of the PNA fraction. It appears, therefore, that non-nucleotide phosphorus-containing compounds occur in the PNA fraction of many organisms and are not peculiar to Gram-positive micro-organisms. There is therefore no evidence to connect the Gram-positive reaction of the micro-organisms examined here with the presence of an 'XSP factor', nor is there need to abandon the previous contention that the Gram staining properties are associated with a complex involving magnesium ribonucleate.

The authors thank Mr E. T. J. Chelton, F.I.M.L.T., for technical assistance and the Medical Research Council for a grant for expenses. One of us (S.B.H.R.) was in the receipt of a Scholarship under the Colombo Plan from the Commonwealth Relations Office, London, for which many thanks are expressed.

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EXPLANATION OF PLATE

Fig. 1. Electrophoretogram of u.v.-absorbing components of PNA fraction of *Klebsiella pneumoniae*. ×0·4.

Fig. 2. Electrophoretogram of the PNA fraction of *Klebsiella pneumoniae*. (×0·3). X = autoradiograph showing components containing ^{32}P; Y = photograph showing u.v.-absorbing components; A = adenylic acid; C = cytidylic acid; G = guanylic acid; U = uridylic acid; P_1–P_{10} = unidentified phosphorus-containing compounds; origin line between P_1 and P_2.

(*Received* 5 *November* 1957)

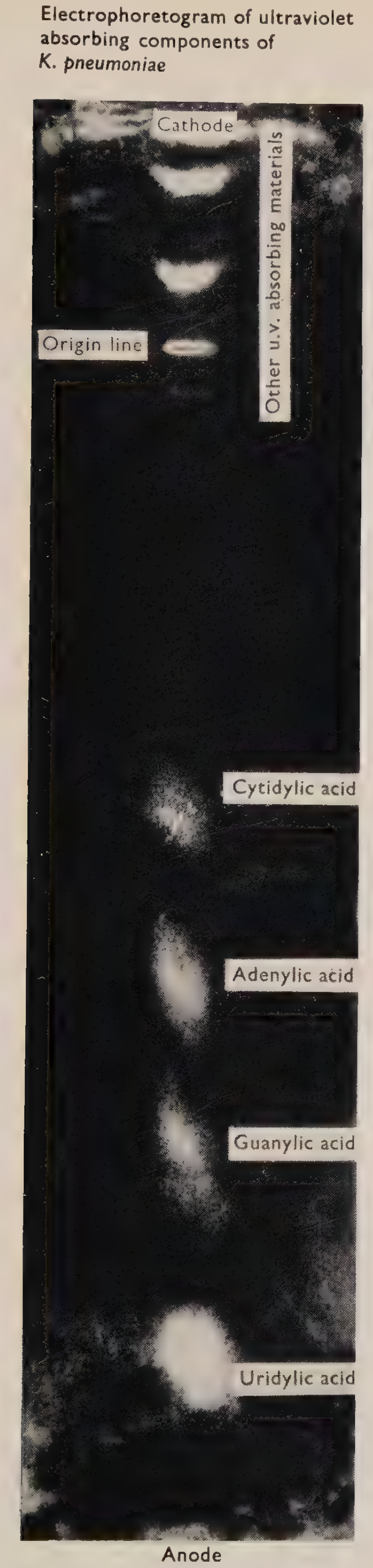

Fig. 1

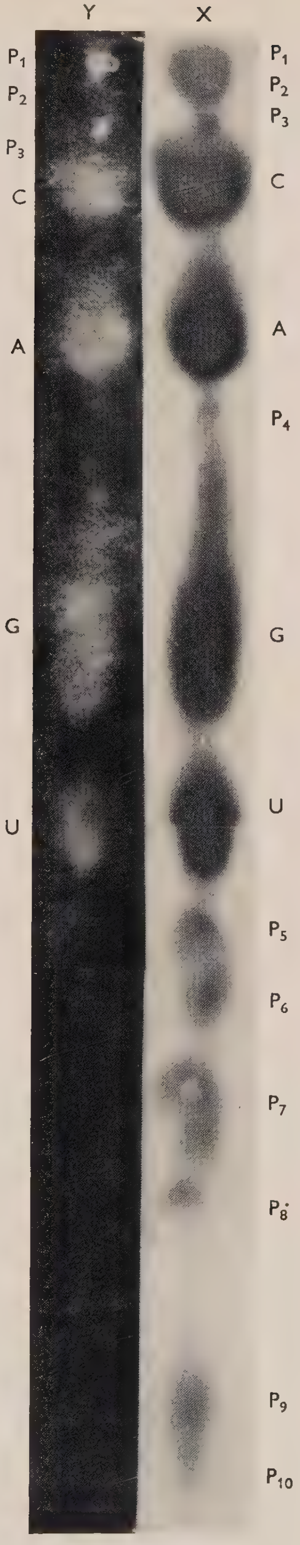

Fig. 2

A. S. Jones, S. B. H. Rizvi and M. Stacey—Role of phosphorus compounds in Gram reaction. Plate 1

(*Facing p.* 606)

Pontieri, G. & Schiano, S. (1958). *J. gen. Microbiol.* **18**, 607–608

Observations upon the Growing Points in Normal and Filamentous *Bacillus megaterium*

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SUMMARY: Normal bacilli of *Bacillus megaterium* have large redox-active granules at the poles of the cells, and smaller ones at the periphery, in the region of the cross-walls. In non-septate filaments produced by growth in the presence of urethane the peripheral granules were not present. This is in accordance with the belief that these are the growing-points associated with the development of the cross-walls.

The 'growing-points' of bacteria were postulated by Bisset (1951, 1952), who maintained that the growth of the bacterial cell and cell envelopes occurred mainly in the areas at the poles and at the junctions of cell wall and cross-wall where basophilic granules were observed. These appear to be the same structures which Mudd (1953) showed to possess redox activity, and which he accordingly equated with mitochondria, although the specificity of these reactions is questionable.

In the present work the occurrence of these granules in normal and in filamentous forms of *Bacillus megaterium* is examined.

METHODS

One strain of *Bacillus megaterium* was used. It was cultivated on nutrient agar plates, and observations were made after 12–14 hr. at 37°. Filamentous forms were produced by addition to the medium of M/30 urethane, according to the technique of Califano & Pontieri (1951).

Observations were made upon the reduction of 2:3:5-triphenyl-tetrazolium (TTC) and of Nadi reagent.

Observations were made with the electron microscope upon material washed in distilled water, suspended in N-NaOH for 1 hr. at 60°, and again washed in distilled water, with the object of increasing the electron transparency of the cell envelopes. The preparations were gold-shadowed. Observations were made with a Philips microscope.

RESULTS

The strain of *Bacillus megaterium* used was isolated in this laboratory; bacilli grown on nutrient agar without urethane were of normal appearance, divided by cross-walls into several cells per bacillus, and showed large redox-active granules at the poles and smaller ones at the periphery, in the regions of the cross-walls (Pl. 1, figs. 1, 2).

In the presence of M/30 urethane the growth consisted of long filaments, without cross-walls, These showed redox activity only in a series of the large granules distributed along the body of the filament in somewhat increased numbers. The smaller peripheral granules were absent (Pl. 1, fig. 3). A similar appearance was shown in the electron micrographs (Pl. 1, fig. 4).

DISCUSSION

The association of the smaller redox-active granules with the cross-walls of *Bacillus megaterium*, and their absence from the non-septate filaments produced by growth in the presence of urethane is in accordance with the view that these are growing-points concerned with the development of the cross-walls. If the larger granules, which are retained, and indeed apparently increased in number, in the filaments, are identical with the polar granules of the septate bacilli, then it is reasonable to conclude that these are the main growing-points of the cell, as suggested by Bisset (1951), and in this case, presumably of the protoplasts. The existence of a granular growing-point in naked protoplasts has recently been described by Jeynes (1957).

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EXPLANATION OF PLATE

Fig. 1. *Bacillus megaterium*, normal bacillus, showing TTC reduction in larger polar granules and smaller peripheral granules in region of cross-walls. Photomicrograph, ×4000.

Fig. 2. *B. megaterium*, electron micrograph, gold-shadowed; normal bacillus; arrows indicate cross-wall. ×10,000.

Fig. 3. *B. megaterium*, non-septate filament grown in presence of urethane, showing TTC reduction only in large granules. Photomicrograph, ×4,000.

Fig. 4. *B. megaterium*, electron micrograph, gold-shadowed; filament grown in presence of urethane, showing absence of cross-walls and series of large granules.

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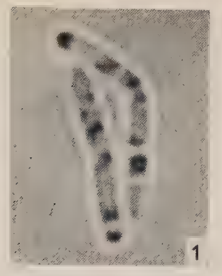

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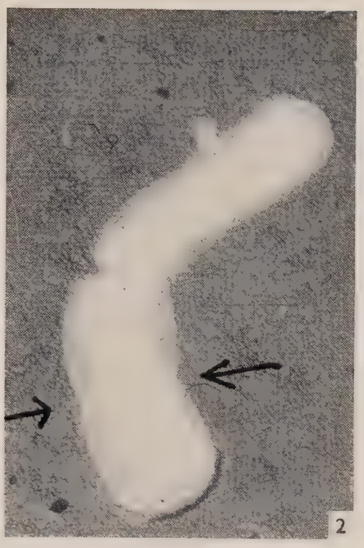

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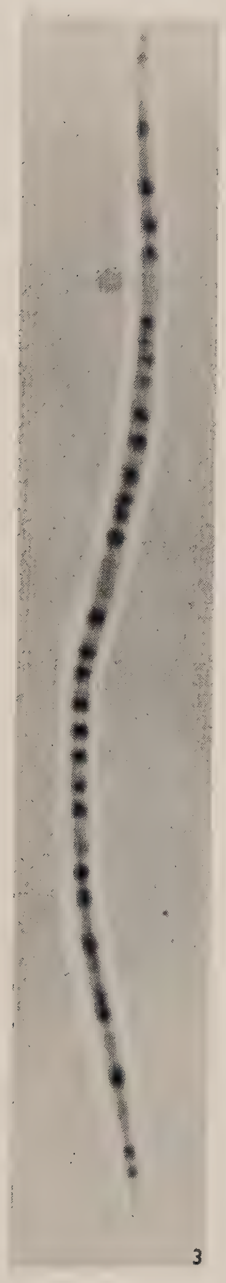

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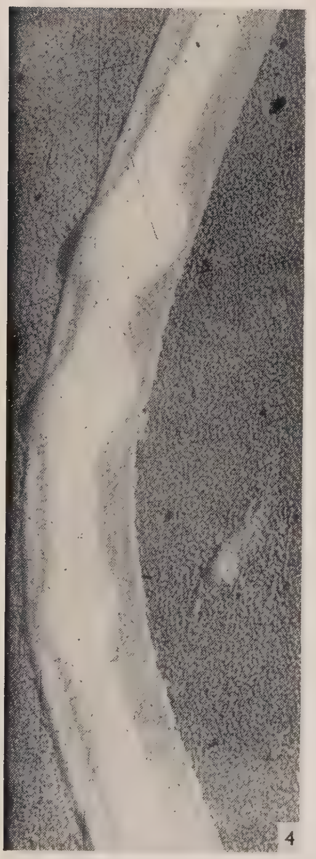

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G. PONTIERI AND S. SCHIANO—MORPHOLOGY OF *BACILLUS MEGATERIUM*. PLATE 1

(*Facing p.* 608)

JANNASCH, H. W. (1958). *J. gen. Microbiol.* 18, 609–620

Studies on Planktonic Bacteria by Means of a Direct Membrane Filter Method

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SUMMARY: Direct observations on the occurrence and spatial distribution of planktonic bacteria were made by using a membrane filter technique. The membrane filters were treated to allow direct microscopic examination of bacteria on their surfaces. Deviations between direct counts on the membranes and plate (colony) counts of bacteria depended upon availability of organic matter in the natural waters studied, and can be accounted for by clumping effects and the occurrence of very small forms not visible on the membrane filter surfaces. Indirect evidence for these 'dwarf' forms was provided by later development of colonies with cells of normal size, after the membrane filter was placed on a nutrient medium. This response suggests that the organisms are zymogenous forms. Pure cultures of *Bacillus subtilis* and *Pseudomonas fluorescens* were used in some experiments to demonstrate the relationship between the clumping effect and concentration of dissolved nutrients in water. A concentration of 0·5 mg. peptone/l. in a tap-water medium caused accumulations of bacteria around clean chitin particles; accumulations of bacteria did not develop at the higher concentrations tested. This behaviour is explained by the local differences in concentrations of nutrients in the medium. It correlates with the observations on natural populations.

The nutritional factor determines not only the distribution of bacteria in natural waters, but also the methods available for their demonstration. Only enriched substrates yield readily measurable bacterial growth and metabolic products in laboratory cultures as well as in nature. The lack of direct indicators of quantity of bacterial growth in media very poor in nutrients may have been a cause for much more work being done on the bacteria of bottom deposits of natural waters as compared to the planktonic forms.

In uncontaminated natural waters the concentration of dissolved organic matter is generally low, but when suitable solid or colloidal organic matter is present, individual bacteria may be confronted with higher concentrations of nutrients. By means of largely unknown and complex adjustments, natural populations of bacteria are able to decompose organic compounds over a wide range of concentration and composition. In the same way that bacterial populations adjust to natural conditions, they adjust to artificial conditions of cultivation. Consequently, it is impossible to determine their original activity after transfer to a medium enriched in nutrients. This means of increasing the formation of reaction products is integral to most of the general methods of estimating the quantity of bacteria. If any process is involved whereby metabolism is promoted, the method should be defined as indirect and the results applied with caution to ecological problems (Gibson, 1957). The use of pure cultures avoids a part of selective adaptation but involves conditions far removed from those affecting natural mixed populations.

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Enumeration of organisms is still the 'most sensible index' (Waksman), and the usual method for assessing the activity of the bacterial population in water or soil, although the relations between growth, cell-multiplication, and respiration may vary. The culture plate (colony count) method is, according to our definition, indirect. In order to employ the advantages of the direct method, the counting of organisms on microscopic preparations, the use of membrane filter techniques is discussed in this paper. A description of some specific applications is given and the results from these are considered in relation to the ecological status of planktonic bacteria.

DIRECT OBSERVATION OF PLANKTONIC BACTERIA

Counts of planktonic bacteria have been obtained by exposing glass slides to the natural medium. Micro-organisms grow on the surface and can be counted microscopically, after preparation, in relation to a unit of exposure time. Previously used for algae (see Lund & Talling, 1957), this method was first applied in soil by Cholodny (1930) and in water by Karsinkin (1934). Although it has been considered a direct method (Henrici, 1933), this view does not accord with the definition above, since the surface properties of the slides promote growth of attaching bacteria and cause a selection of sessile forms (ZoBell, 1943). Finally, this method does not permit an estimate of micro-organisms in a unit area or volume of the original habitat. The difficulty of direct methods is that no distinction is possible between living and dead cells. Nevertheless, experiments by Strugger (1949) on organisms in natural environments, with acridine orange to differentiate between living and dead protoplasm, showed that in the examples studied the percentage of dead cells was low. The rate of growth depends on the concentration of organic matter available, as found by Zavarzina (1955).

The direct methods of enumeration of bacteria in liquids depend mainly upon the population density of the suspensions and the visibility (i.e. size, arrangement and staining reaction) of the individual forms. For most natural waters, a satisfactory means of concentrating the samples is necessary before microscopic observation. Earlier successful approaches were made by Kunznetsov & Karsinkin (1931), who used evaporation under reduced pressure and by Cholodny (1928, 1929), who used filtration, both procedures yielded much higher counts of bacteria than were found by the plating method. The microscopic counting of bacteria in a minute capillary tube of glass after concentration *in vacuo* was described by Collins & Kipling (1957).

The direct membrane filter method

Cholodny (1928) used membrane filters to concentrate bacteria in river water; he streaked the residue on slides. Rasumov (1932) used Cholodny's method but tried to observe the filtered micro-organisms directly on the membrane filter surface. The membrane filter was rendered transparent by immersion oil. This membrane filter technique has been improved and described in detail (Jannasch, 1953; virtually the same technique was reported by Ehrlich, 1955), and was used in the present study. Briefly the membrane filter (from Membranfilter Gesellschaft, Göttingen, Germany) was treated as follows. After filtration of different dilutions of the sample, the organisms on the membrane filter surface were fixed (e.g. with 10% formaldehyde) and stained (e.g. with Löffler's methylene-blue, or phenol (5%)+erythrosin (1%)) by laying the membrane filter on the surface of the staining solution in a Petri dish. The filter material has been decolorized by washing with filtered distilled water to promote an optical contrast. Before each of these three steps, the membrane filter was dried; temperatures up to 90° can be used. By means of a special apparatus the membrane filter surface can be divided into 19 parts, each for the filtration of one sample, or of several dilutions of one sample, at the same time. The prepared membrane filter is then dissected, placed on slides, and rendered transparent by a mixture of caedax (Merck)+liquid petrolatum (ratio 1:1), or liquid petrolatum+α-bromonaphthalene+eugenol (ratio 5:2:2). The refractive indices of these mixtures are adjusted to approach that of the membrane filter used. The standard error of the direct counts has been improved by increasing the number of microscope fields counted. In extreme cases of heterogeneous distribution of organisms, up to 120 microscope fields/membrane filter have been enumerated. A grid of 16 square fields was used at × 800–1000 magnification.

The spatial arrangement of the bacteria in the sample becomes projected on the membrane filter surface. The best results were obtained with pure cultures in the logarithmic phase of growth (Butkevich, 1938), with which the counting error was as low as 4–6% (Jannasch, 1953), due to the even distribution and size of the organisms. In media rich in nutrients, multiplication became apparent before any visible change in optical density occurred.

The general limit of this method, depending on technique and experience, is the visibility of micro-organisms on the membrane filter surface. The main advantage is that concentration of the sample and preparation for microscopic observation are combined in one operation. A valuable feature for field studies is the possibility of fixing and drying the membrane filters by a short process, and storing them for the subsequent preparation and examination in the laboratory.

When the filters are placed on pads of coarse adsorbent paper soaked with a solution of nutrients, this method can be used for cultural purposes (for detailed description see p. 617). For field studies, the pads were soaked in nutrient solution, dried, sterilized in the laboratory. Before use they were saturated with sterile distilled water, conveniently kept in a wash bottle with cotton-wool filter. On ships the rocking motion or the vibration of the engine does not prevent the use of these culture plates.

RESULTS

When the method described was applied to natural waters, and the results compared with those obtained by plating, some interesting relationships appeared concerning the occurrence and spatial distribution of planktonic bacteria. When the bacterial population densities in waters of varied nutrient content were estimated, then with the exception of very clean waters the direct counts exceed the plate counts (Table 1). This seems to be explicable from the microscopic evidence.

In offshore sea water, bacteria were found in groups, often attached to particles (Pl. 1, fig. 1*a*). Here the direct count can be only roughly estimated. As applied in direct counting methods of soil bacteriology (Strugger, 1949), correction factors of 10, 50, or 100 have been used to allow for clumping. Obviously only one colony can be formed on the culture plate regardless of the number of organisms in a clump. These clumps did not occur in the very contaminated sea water studied, where distribution was more homogeneous (Pl. 1, fig. 1*b*) and almost every individual bacterium can be distinguished. In this case the effect of selection may be chiefly responsible for the lower plate count. Samples of contaminated river water showed a similar picture (Pl. 1, fig. 2*b*). When spring water was filtered, however, it was difficult to identify bacteria clearly by their morphological form of arrangment on the membrane filter surfaces (Pl. 1, fig. 2*a*). Different staining methods were used, including differentiation between inorganic and organic material by staining with phenol-erythrosin or water-blue, but without success. When water of the same sample was plated a considerable number of colonies grew. Consequently individuals capable of proliferation must have been present in the spring water. The bacteria of the plate colonies show the normal size and would have been easy to detect on the membrane filter surface.

In the following experiments the two methods were combined to obtain some insight into the early stages of development of these colonies. After filtration of the spring water, a part of the membrane filter was prepared as usual. The remaining part of the membrane filter was laid in a Petri dish on sterile pads of absorbent paper soaked with a sterile solution of the following composition: glucose 150 mg.; peptone 50 mg.; yeast extract 5 mg.; tap water 1 l.; pH 7·2, and incubated at 22°. From hour to hour a part of the filter membrane was removed and prepared. In this way the formation of colonies of only a few organisms (micro-colonies) was observed (Pl. 1, fig. 3). These colonies must have originated from bacterial forms previously not detectable on the membrane filter surface by microscopic examination. The organisms of the micro-colonies were similar in size and shape to those grown on the nutrient agar plates. In some comparable observations, Strugger (1949) isolated coccoid forms of 1 μ. diameter from soil directly, and watched microscopically their development to rods 5–7 μ. long in hanging-drop peptone water culture. Radzimovsky (1930), using Cholodny's direct method, also found very small 'non-countable' bacterial forms in most of the waters he studied. Although in the present experiments the evidence is still indirect, the presence is suggested of very small or 'dwarf' forms, able to develop into larger forms after transfer into nutrient media. From the morphological viewpoint they may correspond to the group of micro-organisms which are defined as 'zymogenous' by Winogradsky (1925).

By means of the same technique they have also been found in other types of clean waters. In Table 1 the number of organisms visible on the membrane filter surface before cultivation has been subtracted from the number of micro-colonies determined after incubation for 6 hr. at 22°. After 10 hr. the colonies begin to grow together (Pl. 1, fig. 3*b*); this may also be a factor tending to decrease the plate counts. The occurrence of dwarf forms, or the formation of micro-colonies in the way described, may have important ecological implications. Their number (see Table 1) appears inversely correlated with the contamination of the water, as estimated (Vinberg & Platova, 1951) by the oxidative determination of reduced material with permanganate. The absence of Gram-positive pleomorphic bacteria was established by Taylor (1942) for natural waters and suggested as the main difference from microflora of soil. The statement could not be confirmed for the types of water studied as shown above.

Table 1. *Counts of bacteria, in various types and zones of natural waters, obtained by plating and by the direct membrane filter method*

The plate counts of sea water were obtained on medium 2216 E, suggested by Oppenheimer & ZoBell (1952). Instead of aged sea water, tap water was used for plating freshwater samples.

	'Rase' (brook)		'Fulda' (river)		'Seeburger See' (eutrophic lake)		Mediterranean Sea (Naples)	
	Continuous spring	Spring brook 1 km. after spring	Rather clean section	Highly contaminated section			Harbour	Offshore sea
Total depth at at station (m.)	0·2	0·15	1·8	2·1	3·8		6·0	1200·0
Depth of sampling (m.)	0·95	0·05	0·5	0·5	0·1	3·7	1·0	1·0
Bacteria as 10^3/ml.								
Plate counts	0·085	18·0	77·0	826·0	1·1	143·0	6,820·0	0·163
Direct counts	0	2·5	289·0	11,230·0	0·52	2600·0	83,270·0	0·341
Count of micro-colonies	0·12	29·0	11·7	0	42·0	1·3	0·06	0·280
$KMnO_4$ consumption in mg./l. 10 min. at 80°								
Sample unfiltered	Trace	4·0	31·6	102·0	20·2	25·0	—	—
Sample filtered through membrane filter	Trace	4·0	28·4	75·5	16·4	11·5	—	—

In addition to the clumping effect in pure waters, a similar feature occurs in waters rich in particulate nutrients. Bacteria were seen growing on the surface of detritus particles which apparently contain amounts of decomposable organic matter (Pl. 2, fig.4). Direct enumeration or, at least, some estimation by counts of organisms in such associations is possible.

Direct observation also readily permits morphological studies. Radzimovsky (1930) and Beling (1950) compared the occurrence of different morphological types of bacteria in river waters of different grades of contamination by cultivation and by direct methods. In all cases the percentage of rods was much higher on the culture plates than in the direct counts. Radzimovsky concluded that more species of coccal forms belonged to the autotrophic and organophobic group, unable to grow on the culture medium used. Besides this selective effect, variations in size and form of the organisms during cultivation may also occur, as shown above. Plate 2, fig. 4*a*, shows irregular coccoid forms frequently to be found in filter preparations of water from the upper layer of an eutrophic lake (the Seeburger See). They were not obtained in various media on culture plates, even Azotobacter-enriching medium being used without success. Unfortunately, the preparation of the membrane filter for direct observation prevents the isolation of individual organisms and further studies of pure cultures. Spirilla, also difficult to grow for quantitative evidence, have been found near or attached to detritus particles (Pl. 2, fig. 4*b*).

OBSERVATIONS ON BACTERIAL AGGREGATES

Aggregation and nutrient supply

The distribution of planktonic bacteria on the membrane filters suggests that the main source of error in the plating method may be the clumping effect. According to ZoBell & Anderson (1936), Stark, Stadler & McCoy (1938), and others, the attachment of bacteria to suspended solid surfaces follows a previous adsorption of soluble organic matter. The effect on the growth of bacteria depends upon the concentration of soluble nutrients present, as shown by Heukelekian & Heller (1940), who measured the amounts of adsorbed organic matter by means of an oxidizing reagent. Some experiments involving a modified membrane-filter technique are described below. They illustrate the influence of small concentrations of dissolved nutrients, in the presence of particulate material, upon the distribution of planktonic bacteria.

Chitin obtained from crab shells was cleaned (Stanier, 1947) and homogenized. Particles of *c.* 10–50 μ. in diameter were obtained by fractional centrifugation. About 500–1000 particles/ml., determined microscopically, were added to sterile-filtered tap water. Organisms from a newly isolated culture of *Bacillus subtilis* were washed, collected on a membrane filter, and added to the suspension of chitin particles in a density of about 10,000 organisms/ml. Parallel cultures were incubated at 22° in 2 l. Fernbach flasks after addition of different concentrations of peptone (0, 0·1, 0·5, 1·0, 5·0, 10·0, 25·0 mg/l.). Continuous shaking prevented sedimentation. No decomposition of chitin by this organism was observed in previous control experiments. At half-day intervals plate counts and direct counts were made.

Three significant concentrations of peptone are chosen in the following description of the findings. The greatest deviation between the plate and direct counts occurred when 0·5 mg. peptone/l. was added (Fig. 1). After 5-6 days the plate count decreased while multiplication was still indicated by the direct observations. The inoculated organisms, at first uniformly distributed on the membrane filter surface, ceased their initial proliferation after about 6 days. Spores began to appear after 8 days in 30–40% of the cells. Basophilic substances inside the cells disappeared, as indicated by vital methylene-blue staining. Between 6 and 8 days bacteria again multiplied, but now on or near the chitin particles. At this stage the membrane preparations appeared as illustrated in Pl. 1, fig. 1*a*. The arrangement of organisms in relation to the chitin particles often suffered some alteration from the filtration process.

In contrast with the effects with 0·5 mg. peptone/ml. no clumping effect was observed with tap water alone, nor in the medium containing 5·0 mg. peptone/l. In both cases the distribution of organisms showed no relationship to the suspended particles; this is illustrated schematically in Fig. 2. The initial multiplication of cells in the tap-water medium without nutrients may be due to reserve substances; the formation of spores here began after only 2 days. A concentration of 5·0 mg. peptone/l. seems to be sufficient for direct uptake from the solution of the offered nutrients by the bacteria. In this case, the difference of nutrient concentrations as between the bulk solution and the particle surfaces may not have been sufficient to show a heterogeneous distribution of the growing bacteria. The existence of a sufficient difference may cause the clumping effect described above.

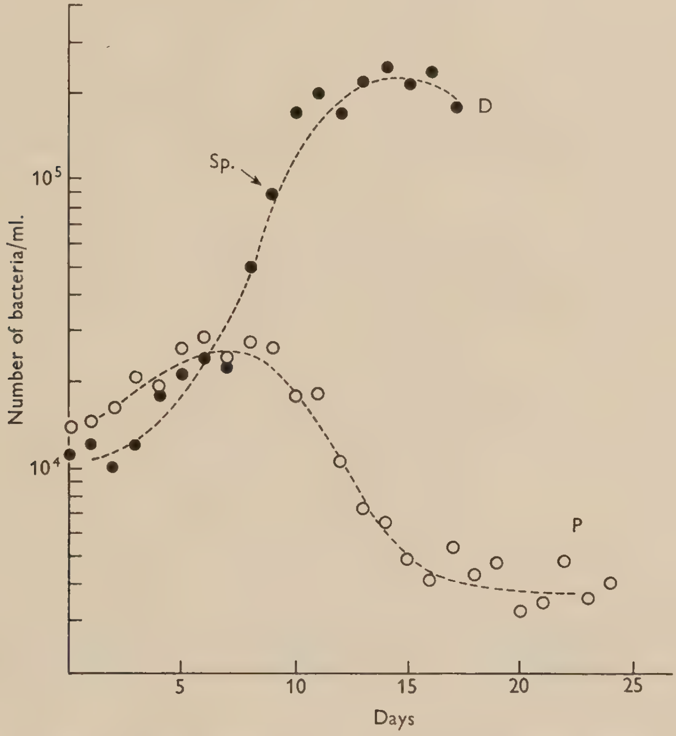
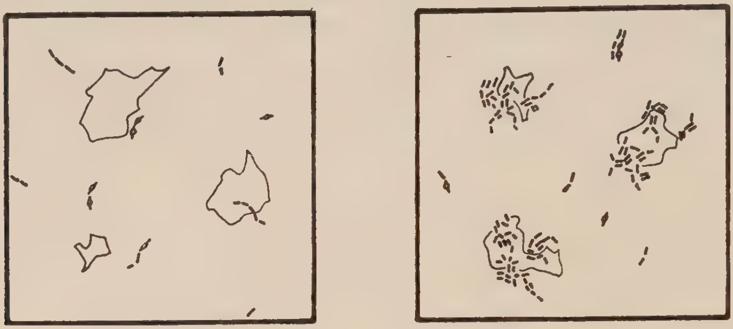


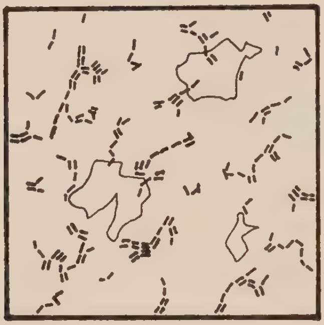
Fig. 1. Direct counts (D) and plate counts (P), per ml., of *Bacillus subtilis* in tap-water media containing 0·5 mg. peptone/l. (Sp. = appearance of spores.)

In several cases growth occurred on suspended chitin particles in media containing only 0·1 mg./peptone/l., but not before 4–5 weeks. Flocculent aggregates appeared in media containing more than 20 mg. peptone/l. and were formed of connected chains of organisms.



No peptone

0·5 mg. peptone/l.



5·0 mg. peptone/l.

Fig. 2. Distribution of cells of *Bacillus subtilis* after incubation for 8 days at 22° in tap-water media containing cleaned particles of chitin and different concentrations of peptone. Schematized from microscopical preparations of membrane filters. Using a grid of 16 fields per square above, the standard errors for the count of the three cultures were 1·2, 5·6 and 0·7%.

Aggregation of particulate material

As compared with the dissolved phase, solid residues of plant and animal tissue must often offer higher concentrations of nutrients to the micro-organisms of a natural medium. There particles may undergo disintegration by the ‘zymogenous’ component of bacterial flora consisting of actively fermenting bacteria and fungi. These organisms may generally exist in a dormant state as spores or their equivalents. If soluble nutrients emerged locally, rapid fermentation processes might occur and large numbers of organisms be formed. After consumption of the energy source, the numbers of vegetative forms would decrease and new resting cells develop. This description was accepted and completed by Conn (1948), especially for the micro-flora of soil. The following experiments (labelled *a*, *b*, *c*) concern the growth of inoculated bacteria in a suspension of particulate nutrients, or of nutrients adsorbed on particulate material. They are intended as 'models' of similar events in natural waters.

(*a*) Suspensions of washed organisms from pure cultures of *Bacillus subtilis* and *Pseudomonas fluorescens*, isolated from water, were stored in tap-water medium, sterilized by filtration, for 4 months. Only a few spores were found microscopically after this period in the culture of *B. subtilis*. Then diatoms, obtained by enrichment culture and killed by heat, were suspended as the sole energy source in the inoculated tap-water medium. The cultures in 2 l. Fernbach flasks were stored at 22° and shaken to prevent sedimentation. The growth of bacteria was observed by means of the direct membrane-filter method.

(*b*) In some parallel experiments, cleaned chitin particles were used instead of dead diatoms. They were enriched nutritionally by exposure for 4–6 days in a nutrient solution of the composition given on page 612, but increased tenfold in concentration. To avoid a rapid diffusion of nutrients into the culture media, the cultivation took place on the membrane filter surface. The enriched chitin particles were filtered together with the suspension of bacteria. The membrane filter was then placed on a paper pad saturated with sterile tap water and incubated under the same conditions as the liquid cultures. After different periods the membrane filters were prepared as usual for microscopic observation.

(*c*) The conditions of the last experiment were modified to test a possible effect of non-enriched particles on bacterial growth. Cleaned chitin particles and the suspension of bacteria were filtered and the membrane filter placed on paper pads soaked with the nutrient solution.

In the liquid cultures (Expt. *a*) of both species of bacteria used, groups of bacteria appeared close to the diatoms after 10–24 hr. When in comparative experiments, acid-cleaned diatom shells were suspended, no bacterial growth was observed. Dense growth appeared after raising the concentration of dissolved nutrients in the water to more than 10 mg. peptone/l., but no clumping occurred, due to the effect of a high enough bulk concentration of nutrient, mentioned earlier.

The same general picture was observed in the membrane filter cultures (Expt. *b*). Rods of the two organisms tested grew next to the chitin particles (Pl. 2, fig. 5) as well as to the diatoms. The membrane filter preparations do not permit the continuous observation of morphological changes in the same colony. Consequently, it was difficult to follow the second process in development of the zymogenous forms, namely the re-forming of the dormant stage. Some observations on this subject have already been described (Jannasch, 1955).

Particles lacking any added nutrients (Expt. *c*) showed no influence upon the distribution of bacteria growing on the membrane filter surface. The bacteria multiplied with a uniform distribution, as shown in Pl. 2, fig. 6. This picture probably corresponds with the third sketch in Fig. 2, although in those experiments the bacterial growth took place in suspension. After a period of 6–8 days, the membrane filters became overgrown and no individual cell could be distinguished.

DISCUSSION

The foregoing observations show that the direct membrane-filter method, as applied in this paper, fails to give total counts; this is due to the occurrence of forms which are difficult to distinguish microscopically on the membrane surface. The development of micro-colonies after addition of nutrients shows that small forms escape observation. Small coccoid forms, on the other hand, are characteristic of the indigenous micro-flora of natural substrates, according to Conn (1948). His definition of the autochthonous (indigenous) group of soil bacteria may be helpful in estimating which type of organism becomes selected and reported by the direct method. He considers the autochthonous micro-flora to include mainly small forms which represent stages in the life cycle of bacteria that are normally rod-shaped (*Arthrobacter*). Similar but non-pleomorphic short rods are collected in the genus *Agrobacterium* Conn. According to Radzimovsky's findings, Conn considers most of the micrococci found in untreated soil as stages of rod-shaped bacteria. Physiologically, the autochthonous bacteria are defined as possessing a weak fermentative ability.

As regards morphological characteristics, all bacterial forms observed directly in pure waters seem to belong to the autochthonous micro-flora. However, the ability of some to develop on concentrated nutrients in a relatively short period would require their classification as zymogenous forms of abnormally small size. From this viewpoint the term 'dwarf forms' seems to be justified. A still unanswered question is whether or not non-spore-forming zymogenous bacteria have a dormant form which is functionally equivalent to a spore. Its existence, even in forms belonging to spore-forming bacteria, is probable, since spores were rarely found on the membrane filter directly when later many colonies grew after cultivation. Despite the limitations of the direct observations, the high counts obtained in often very pure waters suggest that indigenous bacteria are included, although there is no conclusive evidence.

My thanks are due to Dr J. F. Talling for helpful criticism of the manuscript.

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EXPLANATION OF PLATES

The membrane filters were stained with phenyl-erythrosin in Figs. 1*a*, 2*a*, 4*b* and 5; in other figures they were stained with Löffler's methylene-blue.

Plate 1

Fig. 1. Membrane filter preparations of (*a*) offshore sea water, and (*b*) highly contaminated harbour water from the Mediterranean at Naples. The volumes filtered were in the ratio 400:1. (From H. W. Jannasch, 1954, *Naturwissenschaften*, 41, 42.)

Fig. 2. Membrane filter preparation of (*a*) spring water (the Rase) and (*b*) contaminated river water (the Fulda). The volumes filtered were in the ratio 600:1.

Fig. 3. Membrane filter preparations of micro-colonies grown after filtration of spring water and an incubation of (*a*) 5 hr. and (*b*) 10 hr.

Plate 2

Fig. 4. Membrane filter preparations of detritus particles from the upper layers of the Seeburger See.

Fig. 5. Growth of *Pseudomonas fluorescens* on membrane filter close to particles of chitin enriched with peptone.

Fig. 6. Cells of *Bacillus subtilis* uniformly distributed on a membrane filter, which had been placed on paper pads soaked with nutrient solution.

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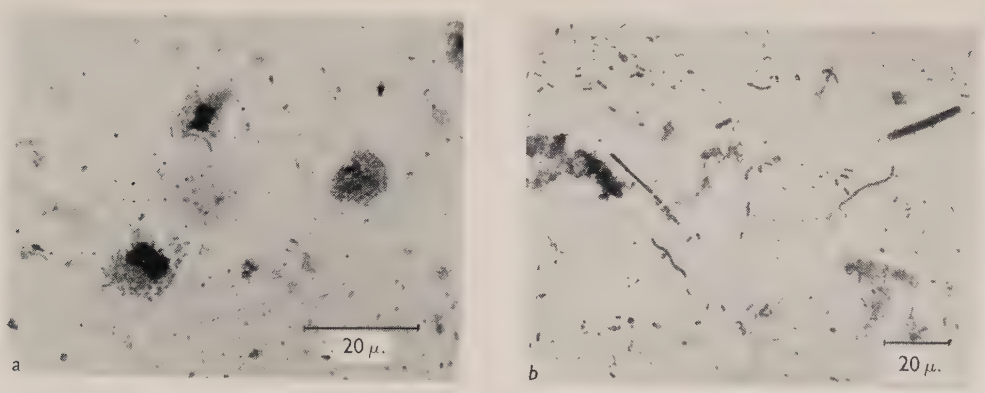

Fig. 1

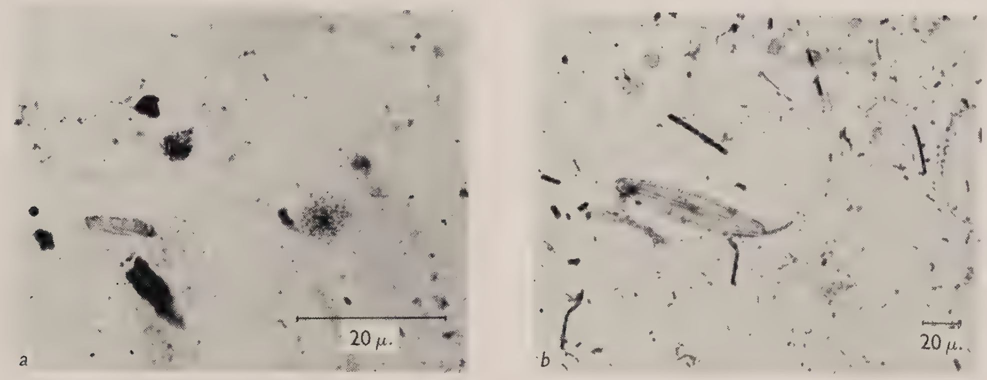

Fig. 2

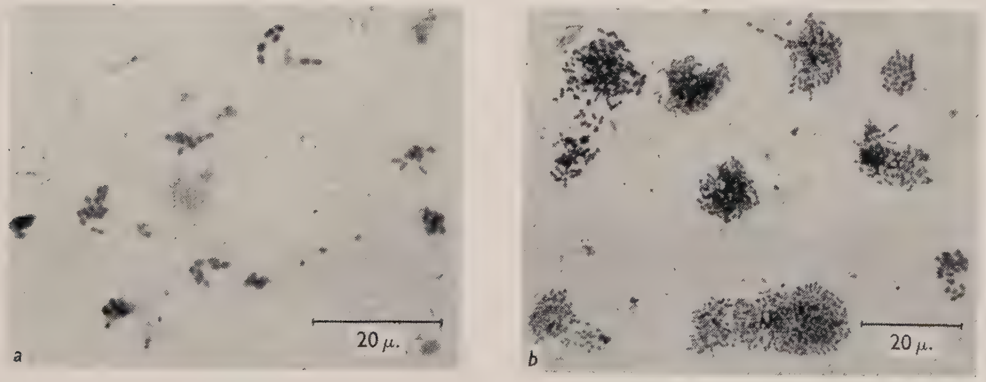

Fig. 3

H. W. JANNASCH—STUDIES ON PLANKTONIC BACTERIA. PLATE 1

(*Facing p.* 620)

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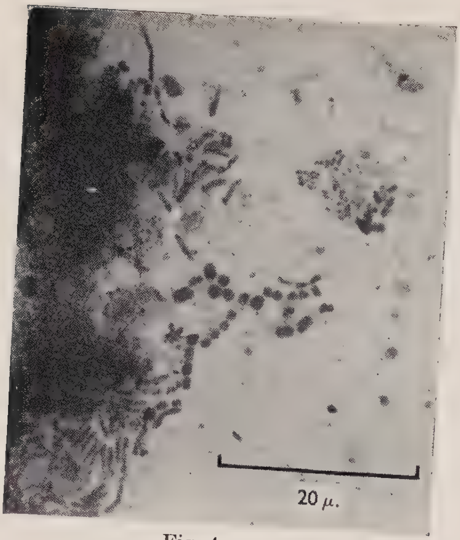

Fig. 4*a*

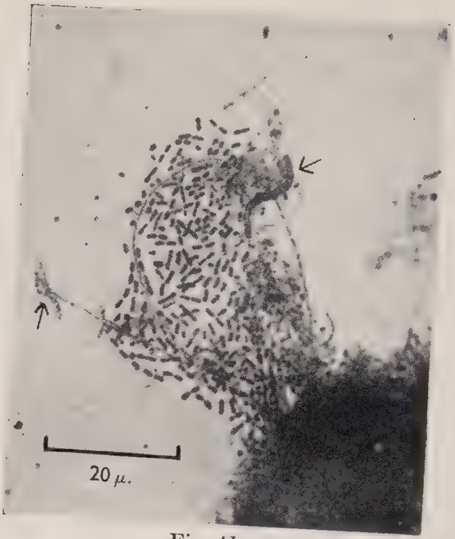

Fig. 4*b*

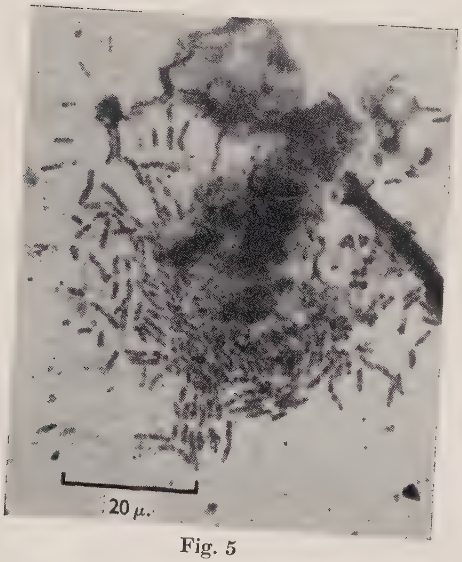

Fig. 5

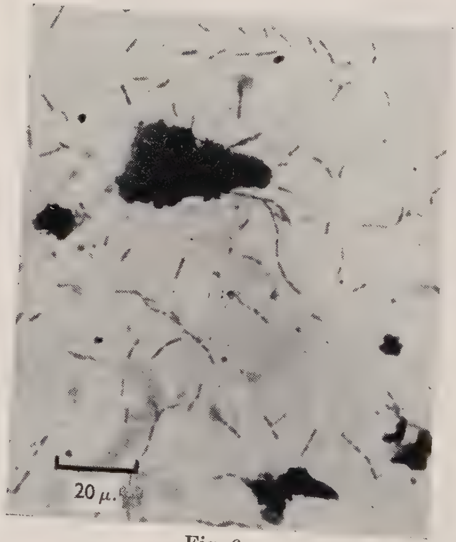

Fig. 6

H. W. JANNASCH—STUDIES ON PLANKTONIC BACTERIA. PLATE 2

KIDDER, G. W. & DUTTA, B. N. (1958). *J. gen. Microbiol.* 18, 621–638

The Growth and Nutrition of *Crithidia fasciculata*

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SUMMARY: The conditions for luxuriant axenic cultivation of the trypanosomid flagellate *Crithidia fasciculata* in chemically-defined media were determined. Improvements over the hitherto published media include higher concentrations of amino acids, folic acid (where this is the sole source of pteridine) and purines and the inclusion of threonine and Tween 80. Increased oxygenation was obtained by incubating liquid cultures in tubes in a sloped position. Under these conditions consistently excellent growth (up to 2×10^8 organisms/ml.) was obtained and the useful incubation period could be decreased to 4 days. Using the improved medium, qualitative and quantitative studies on the nutritional requirements of the organism were carried out. Growth failed when any one of the following amino acids was omitted from the basal medium: histidine, phenylalanine, isoleucine, leucine, valine, lysine, arginine, tyrosine, methionine, tryptophan. The omission of threonine caused drastically decreased growth rates and lower yields. It was found that phenylpyruvic acid could replace phenylalanine; cysteine, cystathionine or homocysteine could replace methionine; and citrulline, but not ornithine, could replace arginine for *C. fasciculata*. In addition to the amino acid requirements a nutritional need for the following was demonstrated: a carbohydrate, haemin, a purine, thiamine, riboflavin, pantothenic acid, nicotinic acid or its amide, pyridoxal or pyridoxamine, biotin, folic acid and an unconjugated pteridine (biopterin). The natural purines, their nucleosides and nucleotides are nearly equivalent, on a molar basis, in growth promotion. Numerous substituted purines were also tested for their ability to supply the purine requirement. No exogenous source of pyrimidine for nucleic acid synthesis is necessary provided folic acid is present. Folic acid can be omitted from the growth medium provided thymine and methionine are present. The absolute requirement for biotin could not be demonstrated in the present medium without the use of avidin.

Crithidia fasciculata, a trypanosomid parasite of the mosquito, has been the subject of a large number of reports. Unfortunately considerable confusion has resulted because of the differences in naming which have appeared (see Wallace, 1943). Biochemical interest in this organism was initiated over twenty years ago when Marguerite Lwoff (1933) showed that it (referred to as *Strigomonas fasciculata*) could be grown in peptone water to which blood or haemin had been added. Recently a number of papers have appeared setting forth cultural conditions and organic requirements of this organism (Cowperthwaite, Weber, Packer & Hutner, 1953; Nathan & Cowperthwaite, 1954; Broquist & Albrecht, 1955). While the Lederle group (Broquist & Albrecht, 1955; Patterson *et al.* 1955) opened up a new field in showing the nutritional importance of an unconjugated pteridine (which they called biopterin) they (Broquist & Albrecht, 1955) report inconsistent growth in the medium of Nathan & Cowperthwaite (1954) which ultimately became so poor as to lose its usefulness (Dr H. P. Broquist, personal communication).

Our attempts to utilize *Crithidia fasciculata* for metabolic studies were hampered by the poor and inconsistent growth obtained in any of the previously published defined media. We therefore undertook a systematic search for the chemical and physical conditions which would permit rapid and abundant growth of the organism. This report describes modified chemically-defined media for *C. fasciculata* which allow for consistent analysis of the nutritional and metabolic patterns of the organism. Using these improved conditions we have examined the quantitative nitrogen and growth factor requirements of *C. fasciculata*. While the qualitative amino acid and growth factor requirements of this organism have been recently described (Cowperthwaite *et al.* 1953; Aaronson & Nathan, 1954; Nathan & Cowperthwaite, 1955; Nathan, Hutner & Levin, 1956), quantitative studies are largely limited to the pteridines (Broquist & Albrecht, 1955; Nathan *et al.* 1956).

METHODS

The strain of *Crithidia fasciculata* used in this study was obtained from Dr H. P. Broquist (Lederle Division of American Cyanamid Co., Pearl River, N.Y.). For stock cultures we used a crude medium consisting of 2% (w/v) proteose-peptone, 0·1% (w/v) Liver Fraction L (Wilson), 1% (w/v) glucose, 0·0025% (w/v) haemin (in triethanolamine), and 0·00025% (w/v) folic acid. This medium was adjusted to pH 8 and was sterilized by autoclaving at 120° for 15 min. While stock cultures can be transplanted at weekly intervals or longer, in practice we found it convenient to make daily transplants so that inoculation organisms of suitable age for experimental use were always available.

In making up the various experimental media, it was found convenient to prepare concentrated solutions of the various components or groups of similar components for final compounding. In this way a medium lacking any desired component could be prepared with ease. The concentrated solutions were layered with toluene and stored at refrigerator temperatures. Tyrosine and guanine, having low solubilities in water, could not be kept in concentrated solutions and were prepared in dilute solutions just before use and added in the desired concentrations. Later this disadvantage was overcome by using tyrosine ethyl ester and guanylic acid, both of which are more soluble. The haemin solution was prepared (at no greater than monthly intervals) in triethanolamine (50%, w/v). When the basal medium was adjusted to pH 8·1 little difficulty was encountered with haemin precipitation.

The basal medium, without glucose, Tween and the compound under investigation, was routinely made up to double strength and distributed in 2 ml. quantities in 15 × 125 mm. test tubes. Appropriate dilutions of the test compound (variable) were added in 1 ml. quantities (in triplicate) to tubes which were aluminium capped before sterilization. Test substances which were unstable to heat were added later after filter sterilization of appropriate dilutions. The glucose and Tween was made up to a fourfold concentration, and after sterilization by autoclaving, the flasks were cooled under running water with shaking to prevent the Tween from separating out. The flask containing the glucose + Tween solution was then inoculated with a 4- to 5-day culture so that the concentration of the inoculum was 1/400 of final medium. One ml. quantities of this inoculated solution were then added aseptically to each tube, bringing the total volume in each tube to 4 ml.

When glucose or Tween was to be tested it was omitted from the standard inoculum and was autoclaved separately and added aseptically to the tubes.

After inoculation the tubes were incubated at 25° in a sloped position. Growth was estimated by determining the optical density at wavelength 650 mμ. by a Lumetron colorimeter.

Crithidia fasciculata tended to settle to the bottom of the tubes and to adhere to their sides. Considerable shaking was required to obtain a uniform suspension for optical density determinations. By coating the tubes with silicone according to the directions given for Siliclad (Clay-Adams, Inc., New York, N.Y.) this tendency was greatly decreased and the time required for making turbidimetric readings markedly shortened.

As the presence of haemin somewhat coloured the medium, uninoculated tubes of the medium under test were used as the blanks when readings were being made. In many experiments the correlation between the turbidity and the number of organisms/ml. was established by comparing optical density with haemocytometer counts.

For the quantitative determinations of the amino acids and of certain of the growth factors, organisms for inoculation could be taken directly from stock medium with no appreciable carry-over. This would indicate low storage in the organism together with relatively high requirements. Preparation of depleted organisms for inoculation was not necessary when carrying out dose-response studies on carbohydrates, haemin, amino acids, purines, thiamine, riboflavin, pantothenic acid and nicotinamide, as essentially no growth resulted in the first transplants when any one of these factors was missing from the medium.

For studies on pyridoxal or pyridoxamine the organisms used for the inoculum were depleted by allowing them to grow in the defined medium without added vitamin B_6 components. When organisms were transplanted from stock medium (even after thorough washing) into such a deficient medium there was appreciable growth due to carry-over, but this growth failed in the second transfer. By using these vitamin B_6-depleted organisms essentially zero blanks were obtained in dose-response experiments, and the organisms responded to added pyridoxal and pyridoxamine in a regular and reproducible manner.

In studies on folic acid and biopterin the preparation of the inoculum was very critical for reproducible results. Storage of these pteridines within the organisms made washing of the inoculum of little use, as fairly good growth resulted in the first transplant to a medium devoid of added pteridines; this situation parallels that with Tetrahymena and folic acid (Kidder & Fuller, 1946). After testing many depletion conditions it was found that the best results were obtained by growing inoculum organisms for 10–11 days in

Medium I (Table 1) in which the folic acid concentration had been decreased to 0·001 μg./ml. While the number of organisms produced (first transfer) was not very different from Medium I minus folic acid, they appeared to be in better physiological condition and responded in a more dependable manner.

Table 1. *Composition of media*

Medium I is the complete medium used in most of the experiments. Medium II is the final medium recommended for *Crithidia fasciculata*. All figures represent μg./ml. final concentration.

Components	Medium I (μg./ml.)	Medium II (μg./ml.)	Components	Medium I (μg./ml.)	Medium II (μg./ml.)
L-Arginine HCl	430	430	Cobalamin	0·001	—
L-Histidine HCl	210	210	Folic acid	1	2
DL-Isoleucine	630	630			
L-Leucine	970	970	Adenine	16	50
L-Lysine HCl	760	760	Guanine	16	—
DL-Methionine	340	340	Xanthine	16	—
L-Phenylalanine	500	500	Uracil	16	—
DL-Threonine	440	440	Cytidylic acid	16	—
L-Tryptophan	120	120			
DL-Valine	660	660	Haemin	25	25
L-Tyrosine	200	—	Triethanolamine	50	50
L-Tyrosine ethyl ester	—	200			
DL-Alanine	550	—	Glucose	10,000	10,000
L-Aspartic acid	610	—			
Glycine	50	—	Tween 80	1,000	5,000
L-Glutamic acid	1,165	—			
L-Proline	770	—	Ethylenediamine-tetraacetic acid	700	700
DL-Serine	440	—			
p-Aminobenzoic acid	3	—	NaCl	4,000	4,000
Biotin	0·2	0·2	Na_2HPO_4	1,250	1,250
Choline Cl	3	3	KH_2PO_4	500	500
Inositol	3	—	$MgSO_4 \cdot 7H_2O$	400	400
Nicotinamide	5	5	$FeSO_4 \cdot 7H_2O$	4·9	4·9
Pyridoxine HCl	2	—	$CaCl_2 \cdot 2H_2O$	7·3	7·3
Pyridoxal HCl	2	2	$Na_2MoO_4 \cdot 2H_2O$	10·1	10·1
Pyridoxamine 2HCl	2	2	$ZnSO_4$	68	68
Ca pantothenate	8	8	$MnSO_4 \cdot H_2O$	77	77
Riboflavin	2	2	$CoSO_4 \cdot 7H_2O$	2·4	2·4
Thiamine	2	2	$CuSO_4 \cdot 5H_2O$	1	1
			H_3BO_3	0·7	0·7
			KI	0·03	0·03

All experiments involving light-sensitive compounds (riboflavin, pyridoxal, pyridoxamine, pteridines and pteridine conjugates) were handled in the absence of white light to minimize their inactivation, and all experimental cultures were incubated in total darkness.

RESULTS

By varying the total amino acid concentration of the medium it was soon found that *Crithidia fasciculata* required a much higher concentration of these components than had previously been used. The original starting-point was the concentrations and proportions used in the studies on *Tetrahymena pyriformis* (Dewey, Parks & Kidder, 1950). The final concentrations found to be satisfactory are given in Table 1, Medium I. With this basal medium, and with appropriate omissions, other components were studied.

It was found that an exogenous source of carbohydrate was necessary for growth, and glucose satisfied this requirement. From dose-response studies it was found advantageous to add 10 mg. glucose/ml. and additional quantities (up to 20 mg./ml.) had no further stimulating effect.

As reported by Marguerite Lwoff (1933), haemin was found to be an absolute requirement for *Crithidia fasciculata*. Growth was in proportion to graded amounts of haemin up to a concentration of 25 μg./ml.; further increases were without effect. Ferrichrome, an iron-chelate compound isolated from *Ustilago sphaerogena*, was reported to replace haemin for the growth of a fungus, *Pilobolus* sp., and was about 500 times more active (Nielands, 1952, 1953). We obtained some ferrichrome from Dr J. B. Nielands (Department of Biochemistry, University of California, Berkeley), but found that it had no haemin-replacing activity for *C. fasciculata*.

Experiments with triethanolamine in concentrations from 1 to 10 mg./ml. showed that it was inert as far as influencing growth was concerned. It is advantageous, however, to include it in the medium as it tends to keep the haemin in solution (Cowperthwaite *et al.* 1953).

Tween 80 was found to be stimulatory to growth (Table 2), the optimum concentration being about 5 mg./ml.; bovine serum-albumin fraction V had about the same effect as Tween. Unlike the case of *Tetrahymena pyriformis* (Kidder, Dewey & Heinrich 1954), where it was found that the apparent stimulation by Tween was not due to increase in number of organisms, actual increase in number of organisms as well as greater optical density occurred with *Crithidia fasciculata* (Table 2). Both Tween and albumin appear to function here by virtue of their physical properties.

Table 2. *Response of* Crithidia fasciculata *to Tween* 80 *and to albumin*

Basal Medium I minus Tween. Four-day growth.

	Tween 80		Albumin	
Addition (mg./ml.)	Optical density	Organisms $\times 10^6$/ml.	Optical density	Organisms $\times 10^6$/ml.
0	0·573	136	0·535	138
1·0	0·683	—	0·647	—
2·0	0·703	—	0·687	—
5·0	0·733	205	0·728	200
10·0	0·730	—	0·722	—

To test the effect of increasing the surface area of the cultures to give better aeration, two sets of tubes containing identical medium and inoculum were incubated together, one set sloped (Kidder & Dewey, 1948) and the other upright. Faster growth and higher yields were obtained in the sloped tubes.

In attempts to simplify the basal medium various changes were made which resulted in Medium II (Table 1). Tyrosine ethyl ester replaced tyrosine.

adenine replaced the nucleic acid derivatives and the non-essential amino acids were omitted. In spite of the sharp decrease in total amino acid concentration no significant decrease in growth rate or total yields resulted. Decrease of the concentration of the essential amino acids, however, decreased growth, so that it would appear questionable whether the non-essential amino acids are indeed utilized. With Medium II (Table 1) average optical densities of 0·720 were obtained by 4 days as compared to 0·060 obtained when the medium of Nathan & Cowperthwaite (1954) was used. An optical density of 0·733 represents over 200 million organisms/ml. (Table 2).

Amino acid studies

When satisfactory growth of *Crithidia fasciculata* was finally obtained attention was turned to specific amino acids. By using Medium I the specific amino acid requirements of the organism were determined by the omission technique. Growth failed when any one of the following amino acids was omitted from the medium: histidine, isoleucine, leucine, lysine, methionine, valine, arginine, phenylalanine, tryosine and tryptophan. This confirms the earlier findings of Cowperthwaite *et al.* (1953). These authors listed threonine as an essential amino acid but later (Hutner & Provasoli, 1955) it was stated that threonine was essential only when adenine was the sole source of purine, but was non-essential when adenosine was added. Nathan & Cowperthwaite (1955) omitted threonine from their medium. We were unable to confirm the relationship between adenosine and threonine synthesis in *C. fasciculata* as we found slow and equal threonine synthesis (as evidenced by growth) in the presence of adenine or adenosine. As can be seen from Table 3, the organisms presumably synthesized threonine slowly and this degree of synthesis was enhanced by the non-essential amino acids. The non-essential amino acid responsible for the stimulation in threonine synthesis is glycine, for essentially the same results were obtained when only glycine was added to the essential amino acid mixture. This may indicate that threonine is synthesized (at least in part) by a reversal of the threonine aldolase reaction (Lin & Greenberg, 1954) and that glycine is one of the limiting factors under these conditions. Under the most favourable conditions for threonine synthesis, however, the organism is very inefficient and the lack of exogenous threonine renders the medium far

Table 3. *Growth of* Crithidia fasciculata *with or without threonine in the presence and absence of the non-essential amino acids*

A = Medium I minus threonine; B = Medium I minus threonine and the non-essential amino acids. Growth indicated by optical density.

	DL-Threonine (μg./ml.)	Days					
		4	5	6	7	8	10
A	0	0·225	0·285	0·354	0·486	0·560	0·620
	80	0·670	0·730	0·747	0·792	0·814	0·921
B	0	0·115	0·161	0·190	0·202	0·250	0·310
	80	0·681	0·735	0·760	0·790	0·800	0·910

from optimal. We are unable to account for the growth obtained by Broquist & Albrecht (1955) on the medium proposed by Nathan & Cowperthwaite (1954) which contained a low concentration of essential amino acids, no non-essential amino acids and no threonine. Using an incubation period of 4–5 days, as they did, we have never been able to get appreciable growth in this medium. Nathan & Cowperthwaite's (1954) growth results are somewhat easier to understand since they used 10- to 18-day growth periods and their turbidimetric unit (Welch Densichron) are over three times that which we use (comparison of the optical density readings of the Welch Densichron and our system was kindly made by Dr S. Aaronson of the Haskins Laboratories), so the high figures given are quite misleading.

α-Aminobutyric acid was said to replace threonine with a tenfold efficiency (Hutner & Provasoli, 1955). When this compound was tested over a wide range of concentrations in the present medium it was found to be without activity in this respect. On the other hand, γ-hydroxy-α-aminobutyric acid (homoserine) substituted readily for threonine.

Homocysteine can replace methionine in the nutrition of *Crithidia fasciculata* (Hutner & Provasoli, 1955). We have found this to be the case in the present medium with or without the addition of a methyl donor (choline, betaine), although added methyl donor improved the transmethylation reaction (Fig. 1). Cystine or cystathionine can likewise replace methionine, so it would appear that the organism possesses the enzymes of the commonly encountered system methionine → cysteine and possibly indicates that the reactions are reversible. While these responses are readily demonstrated in Media I and II, where the folic acid concentration is high, they do not occur when the organisms are growing on a low folic acid and biopterin medium, where methionine becomes an absolute requirement. This probably indicates that the transmethylation is mediated by a folic acid coenzyme, which is to be expected, and that large amounts of the vitamin are required for its formation. High concentrations of biopterin cannot replace the high folic acid requirement for this transmethylation.

Unlike most animal species so far reported, *Crithidia fasciculata* has a requirement for tyrosine as well as phenylalanine (Cowperthwaite *et al.* 1953), although similar requirements have been reported for *Leishmania tarentolae* (Trager, 1956), *Paramecium aurelia* (Miller & van Wagtendonk, 1956) and *P. multimicronucleatum* (Johnson & Miller, 1956). Phenylpyruvic acid can replace phenylalanine for the growth of *C. fasciculata* (Fig. 2), although the keto compound is less active than the amino acid.

When arginine was omitted from Medium I no growth of *Crithidia fasciculata* took place; citrulline replaced arginine for growth and appeared to be more active (Fig. 3). This is completely different from the case of *Tetrahymena pyriformis* (Kidder & Dewey, 1951; Dewey, Heinrich & Kidder, 1957), where it was found that citrulline spared, but never replaced, arginine. Ornithine was not active in *C. fasciculata* in replacing arginine. The fact that citrulline was more active than arginine for growth may suggest a separate function for citrulline, other than as a precursor of arginine.

Many dose-response tests on the ten amino acids required by *Crithidia fasciculata* were carried out. Table 4 gives the amounts of each of these amino acids which had to be added to Medium I (minus the compound being tested) to bring about half maximal and maximal growth. Also included in Table 4 is the amount of threonine ('semi-essential') which must be added to produce maximal growth within a 4-day incubation period.

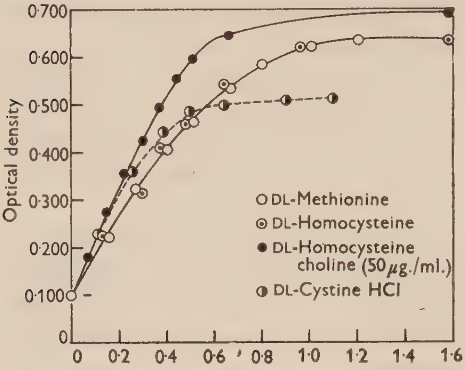

Fig. 1

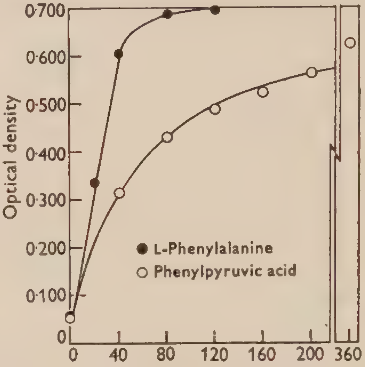

Fig. 2

Fig. 1. Response of *Crithidia fasciculata* to methionine, cysteine, homocysteine and homocysteine plus choline. Basal Medium I minus methionine. Four-day growth.

Fig. 2. Response of *Crithidia fasciculata* to phenylalanine and phenylpyruvic acid. Basal Medium I minus phenylalanine. Four-day growth

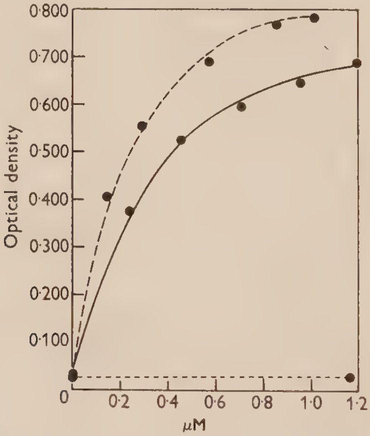

Fig. 3. Response of *Crithidia fasciculata* to arginine, citrulline and ornithine. Basal Medium I minus arginine. Four-day growth. Upper curve (dashed) = L-citrulline; middle curve (solid) = L-arginine HCl; lower curve (dotted) = L-ornithine HCl.

Purines

Crithidia fasciculata has been shown to have a purine requirement (Cowperthwaite *et al.* 1953; Aaronson & Nathan, 1954). This requirement can be fully satisfied by any one of the natural purines (uric acid was not tested), their

Table 4. *Concentrations of amino acids required for half maximum and optimal growth of* Crithidia fasciculata *as determined in Medium I minus the amino acid being tested*

Figures represent μg./ml.

Amino acid	Half maximal	Maximal
L-Histidine HCl	20	80
L-Phenylalanine	16	80
DL-Isoleucine	119	320
DL-Valine	85	200
L-Leucine	30	160
L-Lysine	32	160
L-Arginine HCl	56	350
L-Tyrosine	19	60
DL-Methionine	40	180
L-Tryptophan	7·3	24
DL-Threonine	*	80

* Slow and suboptimal growth without threonine.

Table 5 *Comparison of the activities of free base, nucleosides and nucleotides in satisfying the purine requirement of* Crithidia fasciculata

Figures represent the amount required for half maximum growth in Medium I minus all nucleic acid derivatives. Four-day growth.

	μM
Adenine	0·057
Adenosine	0·063
Adenosine-3′-phosphate	0·057
Adenosine-5′-phosphate	0·061
Guanine	0·062
Guanosine	0·058
Guanylic acid	0·074
Xanthine	0·059
Xanthosine	0·064
Hypoxanthine	0·068
Inosine	0·065

nucleosides or nucleotides (Aaronson & Nathan, 1954); in the present study uric acid was found to be inert. When the natural purines and their nucleosides and nucleotides were compared quantitatively, it was found that only small differences in activities (on a molar basis) existed between them (Table 5). The organism appears to have efficient mechanisms for shifting substituent groupings, both oxidatively and reductively. In order further to investigate these abilities a number of unnatural purines were tested. Those unnatural purines which were not inert expressed themselves by complete replacement of a natural purine or by mild inhibition (Table 6). None showed a sparing action instead of replacement, as is so characteristic of certain purines with *Tetrahymena pyriformis* (Kidder & Dewey, 1949; Kidder, Dewey, Parks & Heinrich, 1950). The purine requirement of *C. fasciculata* was completely satisfied by 6-chloropurine, 2:6-diaminopurine, 6-(2-furfuryl amino)purine (kinetin) and 6-phenylaminopurine, although a time-lag period of greater or lesser extent always manifested itself (Table 7). Certain structural prerequisites appear to exist before this organism can shift substituent groups to form metabolically active purines. No substituted xanthine tested was utilized (uric acid, caffeine, theophylline, theobromine) and no purine substituted in the 8-position (uric acid, 8-methyladenine). No purine unsubstituted at the 6-position could satisfy the purine requirement (purine, 2-aminopurine, 2-hydroxypurine) and the order of substitution in the di-substituted purines was important (i.e. guanine, xanthine and 2:6-diaminopurine were all utilized but isoguanine and 2-methylthio-6-aminopurine were not). One substitution on the amino group of adenine appeared to be successfully removed (kinetin and 6-phenylaminopurine) but not two (puromycin and puromycin nucleoside). Hydrolysis of the chlorine substituent in position 6 (6-chloropurine) appeared to be accomplished with ease but hydrolysis appeared to be impossible when either —SH or —CH_3 was in that position.

Table 6. *Activities of purine and substituted purines for* Crithidia fasciculata

Utilized for growth	Inhibition*	Inert
2:6-Diaminopurine	8-Azaguanine	Isoguanine
6-Chloropurine	6-Mercaptopurine	2-Aminopurine
Kinetin	6-Methylpurine	2-Hydroxypurine
6-Phenylaminopurine	Thioguanine	Purine
	2-Azaadenine	2-Methylthio-6-aminopurine
	1-Deazaguanine	8-Methyladenine
	8-Aza-2:6-diaminopurine	Theophylline
	8-Azaxanthine	Caffeine
	Puromycin	Theobromine
		Uric acid
		Puromycin aminonucleoside

* No compound except 8-azaguanine produced as much as half maximum inhibition when used in concentrations of 200 μg./ml.

Table 7. *Replacement of natural purine (adenine) by unnatural purines*

Basal Medium I minus purine.

	Days			
	2	4	6	8
Purine added at 50 μg./ml.	Optical density			
Control (adenine)	0·370	0·700	0·890	0·950
6-Chloropurine	0·150	0·620	0·840	0·910
6-Phenylaminopurine	0·120	0·480	0·795	0·905
2:6-Diaminopurine	0·047	0·150	0·450	0·895
Kinetin	0·045	0·145	0·455	0·910

It is interesting to note that none of the structural analogues of natural purines tested showed marked inhibition in the growth of *Crithidia fasciculata*. Only 8-azaguanine produced as much as half maximal inhibition and all were tested in concentrations up to 200 μg./ml. The inhibition index (half maximal calculated with guanine) of 8-azaguanine was over 50, which makes it about 1000 times less efficient as an inhibitor for *C. fasciculata* than it is for *Tetrahymena* *pyriformis* (Kidder, Dewey, Parks & Woodside, 1949). Perhaps it is its particular type of purine metabolism which makes *C. fasciculata* almost immune to such generally inhibitory compounds as 6-methylpurine and 6-mercaptopurine.

Aaronson & Nathan (1954) reported that *Crithidia fasciculata* can satisfy its purine requirement with 4-amino-5-imidazolecarboxamidine (the imidazole counterpart of adenine) but not with 4-amino-5-imidazolecarboxamide (the counterpart of hypoxanthine). We have confirmed the inactivity of the carboxamide (the carboxamidine was not re-tested) but we found that the block appears to be in the inability of the organism to add what will become the 2-carbon of hypoxanthine, for 4-formylamino-5-imidazolecarboxamide will satisfy the purine requirement. This compound is not utilized very efficiently as rather high concentrations are needed for maximal growth in the absence of other purines. Filter sterilization was used for this compound as it is known that spontaneous ring closure can take place at high temperatures. Lack of ability of *C. fasciculata* to close the imidazole ring to form a purine is shown by the fact that both 2:5:6-triamino-4-hydroxypyrimidine and 2:6-diamino-4-hydroxy-5-formylaminopyrimidine are inert for this organism.

Vitamins

Cowperthwaite *et al.* (1953) listed seven vitamins as being required for the growth of *Crithidia fasciculata* but they gave no experimental details. We have confirmed their qualitative findings and have studied the quantitative aspects of the problem. In the present medium, and with care regarding depletion of the organisms for inoculation (see Methods), growth failed when thiamine, riboflavin, nicotinamide, pyridoxal and pyridoxamine, pantothenic acid or folic acid was omitted from the medium. When catalytic amounts of folic acid were present then biopterin had to be added.

Indefinitely transplantable, though suboptimal, growth occurred in media to which no biotin was added. This would indicate that some component of the medium contained biotin as a contaminant, or that the organism was synthesizing suboptimal amounts of the vitamin, or that end-products of biotin-controlled reactions were present in the medium. The last possibility was tested by the omission of aspartic acid, Tween 80 (which contains oleate) and acetate. Their omission did not influence the serial transplantability of *Crithidia fasciculata* in medium with no added biotin. It seems probable that there was some biotin contamination of the haemin used, since decreasing the haemin content of the medium decreased the amount of growth, and this growth was restored, within limits, by adding biotin (drastic decrease of the amount of haemin brought about a haemin deficiency which was not annulled by adding biotin). The inclusion of crystalline avidin, however, made growth impossible and this inhibition was entirely annulled by adding biotin. It seems safe to conclude that *C. fasciculata* is dependent upon an exogenous source of biotin, for the binding power of avidin is not exerted on endogenous biotin.

In Table 8 the quantities of the various vitamins required for half maximal growth are given. It will be seen that nicotinamide was about five times more active than the acid, and that pyridoxamine was about twenty times more active than pyridoxal. Pyridoxine was without activity.

Table 8. *Quantitative requirement of vitamins for* Crithidia fasciculata *in Medium I (minus appropriate vitamin)*

Results read after 4 days of incubation where maximum growth was represented by optical densities of 0·670–0·740.

Vitamin	Amount required for half maximum growth (μg./ml.)
Thiamine	0·011
Riboflavin	0·015
Ca pantothenate	0·046
Nicotinamide*	0·065
Nicotinic acid*	0·295
Pyridoxal HCl†	0·019
Pyridoxamine 2HCl†	0·0035
Folic acid‡	0·0004
Biopterin§	0·0001

*Nicotinic acid or nicotinamide active alone.

† Pyridoxal or pyridoxamine are active alone.

‡ Tested in the presence of 0·005 μg./ml. of biopterin (2-amino-3-hydroxy-6-trihydroxypropyl pteridine).

§ Tested in the presence of 0·03 μg./ml. of folic acid.

While folic acid alone will support the growth of *Crithidia fasciculata* (Cowperthwaite *et al.* 1953) relatively extremely high concentrations are required. On the other hand, high concentrations of biopterin (with no folic acid present) did not support growth. When the two were present together then low concentrations of each produced maximal growth (Table 8; Fig. 4). A comparison of the activities of various 2-amino-4-hydroxy-6-alkylpteridines (these compounds were kindly supplied by Drs H. P. Broquist and E. L. R. Stokstad, Lederle Division, American Cyanamid Co., Pearl River, N.Y.) for biopterin activity disclosed that the one isolated from urine (Patterson *et al.* 1955) and the synthetic biopterin (2-amino-4-hydroxy-6-erythrotrihydroxypropyl pteridine) were equal, but that 2-amino-4-hydroxy-6-(D-threo-1′:2′-dihydroxypropyl)pteridine (sent by Professor M. Viscontini for activity assay) had only slight biopterin activity. The HB_2 Drosophila pteridine of Viscontini, Loeser, Karrer & Hadorn (1955; sent by Professor M. Viscontini) proved to be four times more active than the biopterin from urine. When the trihydroxypropyl biopterin is oxidized in position 7 (7-oxybiopterin, supplied by Professor R. Tschedsche) then all activity was lost. These observations add to our knowledge of the specificity of the molecule for biopterin activity beyond that which was previously published by Patterson *et al.* (1955).

It appears that *Crithidia fasciculata* is very inefficient, but not entirely deficient in mechanisms for cleaving a conjugated pteridine to an unconjugated pteridine but has no ability to construct folic acid. This is borne out by the observation that pteroic acid can supply the biopterin requirement if

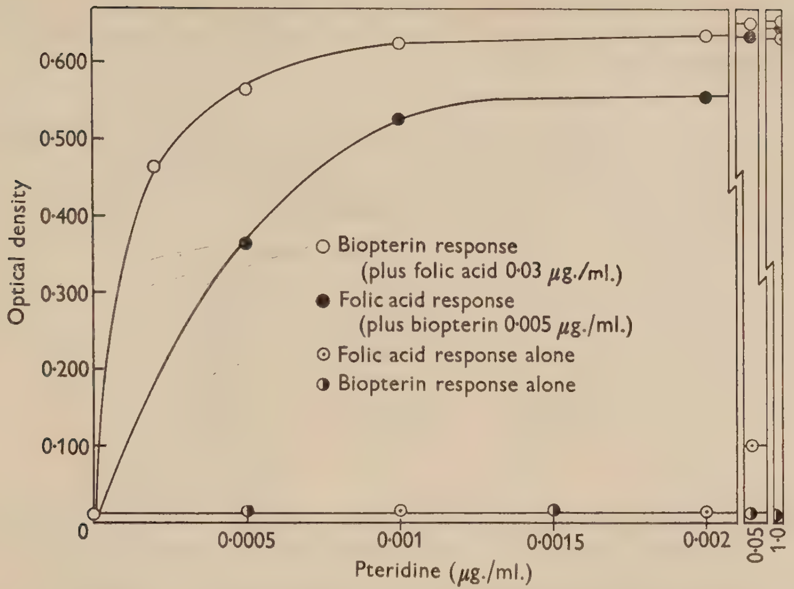

Fig. 4. Dose response of *Crithidia fasciculata* to biopterin (2-amino-4-hydroxy-6-erythro-trihydroxypropyl pteridine) and to folic acid. Inoculum was from 10-day culture in low (0·001 μg./ml.) folic acid and no biopterin.

Table 9. *Growth response of* Crithidia fasciculata *to folic acid and pteroic acid in the presence and absence of biopterin*

Four-day growth.

Amount added (μg./ml.)	Optical density			
	Folic acid	Pteroic acid	Folic acid + biopterin*	Pteroic acid + biopterin*
0	0·033	0·027	0·044	0·043
0·001	0·038	0·026	0·495	0·054
0·01	0·052	0·034	0·543	0·297
0·1	0·290	0·392	0·580	0·425
1·0	0·577	0·543	0·633	0·578

* 0·0003 μg./ml.

present in high concentrations (Table 9), but cannot act catalytically with added biopterin, as folic acid does. Growth with pteroic acid and biopterin is limited to the contamination of the pteroic acid with small quantities of folic acid (traces of folic acid were found by the use of paper chromatography in all of our samples of pteroic acid) when low levels are used (Table 10).

As reported by Nathan *et al.* (1956), the folic acid requirement can be by-passed by the addition of thymine or thymidine, provided methionine (not homocysteine, etc.) is present in the medium. The optimum amount of thymine or thymidine which had to be added to a biopterin-containing medium was about 25 μg./ml., as the nucleoside was about twice as active as the free base on a molar basis. With either thymine or thymidine growth never reached maximal values (Table 11), and the degree of growth did not change with longer incubation periods.

Table 10. *Growth response of* Crithidia fasciculata *to increasing amounts of biopterin in the presence of folic acid and pteroic acid*

Four-day growth.

Biopterin (μg./ml.)	Optical density Folic acid 0·005 μg./ml.	 Pteroic acid 0·005 μg./ml.
0	0·022	0·013
0·0001	0·390	0·260
0·0002	0·570	0·200
0·0005	0·630	0·210
0·1	0·684	0·213

Table 11. *Growth response of* Crithidia fasciculata *to increasing amounts of biopterin in the presence of folic acid, thymine and thymidine*

Basal medium contained optimal amounts of methionine, serine and purine. Five-day growth.

Biopterin (μg./ml.)	Optical density Folic acid, 0·002 μg./ml.	 Thymine, 40 μg./ml.	 Thymidine, 40 mg./ml.
0	0·016	0·052	0·031
0·0001	0·270	0·337	0·382
0·005	0·670	0·394	0·431
1·0	0·687	0·437	0·467

Following the suggestion of Nathan *et al.* (1956), we tested the effect of high concentrations of riboflavin on the biopterin requirement of *Crithidia fasciculata*. In the present medium riboflavin at as high as 5–10 μg./ml. had a slight sparing effect on biopterin, but as both compounds were absolute requirements and the sparing effect was low, it seems improbable that the scheme presented by Nathan *et al.* (1956) for the interrelationships of the two can be fully accepted.

DISCUSSION

As previously stated, we were unable to obtain satisfactory growth of *Crithidia fasciculata* in the medium proposed by Nathan & Cowperthwaite (1954). It now is apparent that the deficiencies of this medium are multiple. The total amino acid concentrations are too low for this organism, and we have had to add over five times the concentration of the essential amino acids that was suggested (Table 12). The omission of threonine from their medium brings about a very serious deficiency. Less important differences between their medium and ours (Medium II) include suboptimal purine, folic acid and isoleucine and the absence of Tween.

After the above experimental work was completed we have used the inorganic salt concentrations suggested by Newton (1956) and have thereby been able to simplify that portion of the present medium. While there is slight precipitation of the salts, as this mixture contains no ethylenediaminetetraacetic acid, growth of *Crithidia fasciculata* was excellent.

Table 12. *Comparison of amino acid concentrations* (μg./ml.) *of the medium of Nathan & Cowperthwaite* (1954) *with those of the present Medium II*

Amino acid	N. & C.	Medium II
L-Arginine HCl	250	430
L-Histidine HCl	200	210
DL-Isoleucine	50	630
DL-Leucine	50	—
L-Leucine	—	970
DL-Lysine	200	—
L-Lysine	—	760
DL-Methionine	80	340
DL-Phenylalanine	40	—
L-Phenylalanine	—	500
DL-Tryptophan	20	—
L-Tryptophan	—	120
L-Tyrosine	50	—
L-Tyrosine ethyl ester	—	200
DL-Valine	50	660
DL-Threonine	—	440
Total	990	5260

The nutritional pattern of *Crithidia fasciculata* regarding amino acids differs only in minor details from that of other animals. The ability to replace methionine with cysteine and cystathionine is unusual, although the ability to transmethylate homocysteine is widespread. This transmethylation is not possible in *Tetrahymena pyriformis* (*geleii*) (Genghof, 1949). The fact that citrulline, but not ornithine, can replace arginine for this organism makes it similar to certain stocks of Drosophila (Hinton, Noyes & Ellis, 1951; Hinton, 1956) and quite different for *Tetrahymena pyriformis* (Dewey *et al.* 1957).

The requirement for a pre-formed purine has been found to be rather widespread among bacteria, moulds and protozoa. In the latter group a particular purine is often needed (Kidder & Dewey, 1949; Kidder *et al.* 1950; Kidder & Dewey, 1955; Miller & Johnson, 1957) while purine-requiring bacteria most frequently will utilize any one of a number of purines (Pearson, 1949; Guthrie, 1949; Gots, 1957). In this respect, therefore, *Crithidia fasciculata* resembles the bacterial types rather than the ciliates. We do not know at present the specific pathways used by *C. fasciculata* for the interconversions of purines, but it is obvious that they can both reduce and oxidize at position 2 on the ring, provided position 6 is correctly substituted. A curious situation exists regarding isoguanine (2-hydroxy-6-aminopurine). Mechanisms appear to be absent for handling this compound, although 2:6-diaminopurine and xanthine are readily used. Gots (1957) reported the exact reverse situation in a purineless mutant of *Escherichia coli* where isoguanine, but not 2:6-diaminopurine was utilized.

There appears to be no lack of functional enzymes in *Crithidia fasciculata* for attaching and removing sugar and sugar phosphates, as bases, nucleosides and nucleotides are all about equally active. At the present time there is no indication as to the stage (base, nucleoside, nucleotide) at which the shifting of substituent groups on the purine ring occurs.

The qualitative requirements for the water-soluble vitamins are not unusual except for the pteridines. It should be pointed out that *Crithidia fasciculata* resembles the ciliates (Kidder, Dewey, Andrews & Kidder, 1949) in its failure to synthesize nicotinic acid from tryptophan, for both the amino acid and the vitamin are absolute requirements.

It would appear that the enzymes necessary for the cleavage of folic acid to the unconjugated pteridine are very inefficient, so that extremely high concentrations of the conjugated forms (folic acid and pteroic acid) must be present before active levels of biopterin can be produced. There can be no question of the importance of a folic acid coenzyme function, however, as distinct from a biopterin coenzyme function, for no amount of biopterin will permit growth in the absence of thymine or methionine. Even when both of these common products of one carbon transfer reactions are provided, growth is suboptimal. It seems probable, as suggested by van Baalen, Forrest & Myers (1957), that neither the dihydroxypropyl nor the trihydroxypropyl biopterins are the naturally active forms of the unconjugated pteridines, but more active forms can be synthesized from them by this organism. The HB_2 Drosophila factor, for instance, is four times as active as the biopterins on a weight basis and its molecular weight must be larger, not smaller.

At present there is no evidence as to the metabolic function of the unconjugated pteridines, but their widespread occurrence in nature would suggest that they will ultimately be found to have great metabolic significance. Inasmuch as *Crithidia fasciculata* has a requirement for unconjugated pteridines, and is the only organism so far known with such a requirement, it may now be employed for a study along these lines.

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The Cytology of *Pseudomonas* spp. as Revealed by a Silver-Plating Staining Method

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SUMMARY: A modification of Fontana's silver-plating method was devised and used for the cytological study of 214 isolates of *Pseudomonas* spp. Many features, such as flagella, cross-walls or septa, capsules and slime were thereby clearly demonstrated simultaneously and easily. Comparisons were made with other published methods for staining flagella, capsules and slime, and good agreement was obtained. No totally non-flagellate isolate was found, nor were flagella lost during maintenance for 3–5½ years in the laboratory. The number of flagella/rod and also bipolar flagellation were found to be characters of little value for species differentiation. The flagellation of young cultures was not found to be significantly different from that observed in older cultures; the polar position of the flagella was confirmed. Flagella curvature (wavelength and amplitude) as seen in silver-plated organisms prepared under reasonably standardized conditions, was noted and considered to have no usefulness for subgeneric classification.

The generic identification of *Pseudomonas* spp. presents little difficulty when the organisms produce the characteristic pigments, but achromogenic strains are common and often not easily distinguishable from *Acetobacter*, *Vibrio*, *Alcaligenes* and *Achromobacter* spp. (cf. Shewan, Hodgkiss & Liston, 1954). Since *Alcaligenes* and *Achromobacter* are, by family definition, either non-motile or peritrichously flagellate, a simple method to determine flagellation would be of value. The separation of *Pseudomonas* and *Chromobacterium* can also be difficult, but the work of Sneath (1956*a*, *b*) on the latter genus greatly facilitates the diagnosis. Detailed studies of *Chromobacterium* flagellation were also reported by Sneath (1956*b*), and his results will be discussed later.

The position and number of flagella/organism are cited in *Bergey's Manual* (1948) as part of the definitions of *Pseudomonas* species; these are summarized in Table 1. Therefore it was thought desirable to investigate also the degree of variability in the number of flagella/organism, in order to ascertain the usefulness of this criterion for species differentiation, bearing in mind the value of this character in the classification of algae and phycomycetes. Leifson (1951) suggested, after much experience involving observations of flagella-stained preparations of a large number of different kinds of bacteria, that the degree of curvature (i.e. wavelength and amplitude) of bacterial flagella was perhaps significant, since this character appeared usually to be constant for a given strain; he claimed that in some cases this detail merited consideration even for species or genus definitions. Because many more observations with large numbers of strains are obviously needed, details of flagellation were carefully recorded in the work now to be described.

Table 1. *Summary of the flagellation of the* 149 Pseudomonas *species (excluding appendices) as listed in* Bergey's Manual (1948)

	Species		Species
One polar flagellum	42	4–6 polar flagella	1
1–2 polar flagella	13	5–7 polar flagella	1
1–3 polar flagella	12	Motile and lophotrichous	1
1–4 polar flagella	9	Motile and polarly flagellate	15
1–5 polar flagella	12	Motile (presumably polar flagellation)	2
1–6 polar flagella	5	Motile	9
1–7 polar flagella	1	Non-motile	6
1–several polar flagella	8	Not stated	1
2 polar flagella	1	Anomalous (monotrichous or 1–3)	1
2–4 polar flagella	5		
2–5 polar flagella	2		
2–6 polar flagella	1		
2–8 polar flagella	1		

METHODS

Media. The organisms used were maintained in yeast-extract liquid medium or on this medium solidified with agar. The yeast-extract medium had the following composition (%, w/v): Oxoid bacteriological yeast extract, 0·3; peptone (Evans), 1·0; NaCl, 0·5; adjusted to pH 7·2–7·4 with NaOH. For solid medium 2·0% (w/v) of agar was added.

Growth temperature. All cultures were incubated at 25°, although this was not necessarily the optimum temperature for the growth of every strain.

Source of organisms examined. Two hundred and fourteen isolates of aerobic Gram-negative rod-shaped bacteria, considered likely to be pseudomonads on the basis of various cultural and biochemical characters such as nutrition, temperature range, oxygen requirements, colonial morphology, proteolytic, saccharolytic and lipolytic properties, were obtained from a wide variety of natural sources (human blood and wounds, diseased plants, soil, milk, sewage, many different kinds of water). Nineteen cultures of plant pathogenic *Pseudomonas* spp. were supplied by Dr W. J. Dowson (Cambridge), and five named cultures were obtained from the National Collection of Type Cultures.

Purification of isolates. Isolates were purified by alternate subcultivation of single colonies in yeast-extract liquid medium and streaking on yeast-extract agar, the process being repeated at least five times.

The staining of flagella

A modification of Fontana's method for staining spirochaetes (Mackie & McCartney, 1949, p. 108) was devised, the modifications consisting of slight differences in the reagents and in the method of preparing bacterial films for staining. Two reagents were used:

(1) *Ferric tannate mordant.* To 10 ml. 10% (w/v) tannic acid were added 5 ml. saturated aqueous potash alum, followed by 1 ml. saturated solution of aniline in water; the curd which formed was re-dissolved by shaking. The

addition of 1 ml. 5% (w/v) ferric chloride gave a black solution which was allowed to stand 10 min. before use.

(2) *Ammoniacal silver nitrate solution.* From 100 ml. of a 5% (w/v) aqueous solution of silver nitrate *c.* 10 ml. were set aside. Concentrated ammonia solution (sp.gr. 0·880) was slowly added to the 90 ml. portion until the brown precipitate just re-dissolved. Drops of the 10 ml. sample of silver nitrate solution were then added until the solution remained faintly cloudy, even after shaking. Stored in the dark, this solution remained stable for several weeks.

Method of making a preparation and staining to show flagella. Organisms were grown on yeast-extract agar slopes in 6 × $\frac{5}{8}$ in. tubes, to each of which was added 1–2 ml. sterile distilled water because motile organisms were most readily obtained from the liquid at the base of such slopes. Flagella staining of these cultures was made routinely after 18–24 hr. of incubation, or after 48 hr. in the case of the few cultures which grew slowly, e.g. *Pseudomonas lachrymans.* After incubation, two 4 mm.-loopfuls of the liquid at the base of a slope were inoculated into 5 ml. sterile distilled water and incubated for 1 hr. (25°) to remove débris and excess mucilage from the organisms. A drop of this suspension was then transferred to a slide previously cleaned in chromic + sulphuric acid mixture. After allowing the drop to air-dry, the film was covered with the iron tannate mordant reagent (no. 1) for 3–5 min. After very thoroughly washing the film with distilled water the silver reagent (no. 2), heated nearly to boiling, was applied and left in contact with the film for 3–5 min. After again washing the film with distilled water, it was blotted, and either examined directly or made permanent under a coverslip by sealing with neutral Canada Balsam. Exposure to air caused disintegration of these silver-plated preparations after about 1 week. A light microscope with $\frac{1}{12}$ in. oil-immersion objective and × 10 ocular was used to examine the preparations. The silver-plated flagella were found to be easily visible provided that the sub-stage condenser had been carefully centred and focussed. From personal experience it has been found that lack of attention to this technical detail has led many students to believe that flagella staining is difficult. The author found that focusing (under a $\frac{2}{3}$ in. objective) a few threads of cotton wool held in front of the microscope lamp was a simple method for obtaining the necessary optical efficiency.

Each of the *Pseudomonas* isolates collected was examined at least three times by this flagella-staining technique, after various periods of maintenance (up to 5½ yr.) as laboratory stock cultures preserved on yeast-extract agar slopes covered with mineral oil (Rhodes, 1957).

Yeast-extract agar + water slope cultures of twelve isolates selected at random were examined by the routine silver-plating technique at hourly intervals up to 18 hr. after inoculation of old non-flagellate cultures onto fresh slopes. Thus were investigated the reports of Leifson & Hugh (1953) and Sneath (1956*b*) that lateral flagella were found in young cultures of bacteria (*Aeromonas* and *Chromobacterium* spp.), but that these cultures yielded only polarly flagellate organisms after longer incubation.

RESULTS

Flagellation, slime and capsule formation in 214 pseudomonad isolates

On each slide preparation bearing organisms from one psuedomonad isolate, 200–1000 individual organisms were examined and on many of them polar flagella were frequently clearly visible (see Pl. 1 and Fig. 1). Although occasionally all the organisms in the preparation from a given isolate appeared to be non-flagellate, this was often due to the presence of an excess of slime which spoilt the film: in all these cases a subsequent more dilute preparation, or a slightly older or younger one, showed some polarly flagellate organisms. Thus not one totally non-flagellate isolate of *Pseudomonas* was found in the collection examined.

Number of flagella/organism

Table 2 summarizes the results of some of the routine examinations for numbers of flagella, and shows that in the great majority of cases the number of flagella/organism was extremely variable in any given slide preparation.

Table 2. *Analysis of the silver-plated preparations of* 150 *isolates of* Pseudomonas *to show the variation in the number of flagella/organism*

Each preparation was made from a drop of a very dilute aqueous suspension of organisms, and each isolate was examined three times (at various intervals over 5½ yr.)

Slide preparations showing	Number of slide preparations examined
All organisms with same number of flagella	90
Organisms with two different numbers of flagella	106
Organisms with three different numbers of flagella	112
Organisms with four different numbers of flagella	89
Organisms with five different numbers of flagella	32
Organisms with more than five different numbers of flagella	14
No flagellate organisms	7
	450

On repeat staining of the 214 isolates the same numbers of flagella/organism as previously recorded were found only for 17 isolates; the remaining 197 isolates showed different values for flagella/organism at the repeat. Plate 1, figs. 1 and 3, show typical microscope fields of preparations in which the different flagellar numbers are clearly visible. In general, rods with small numbers of flagella (1–3) were characteristic, but up to 10 flagella/organism were not uncommon (see Fig. 1 and Pl. 1).

The position of the flagella

In about 95% of the individual flagellate organisms examined, the flagella were clearly seen to originate from the rounded polar regions of the rods. Flagella which appeared to be lateral were occasionally observed but, on careful examination, it could often reasonably be interpreted that the dense

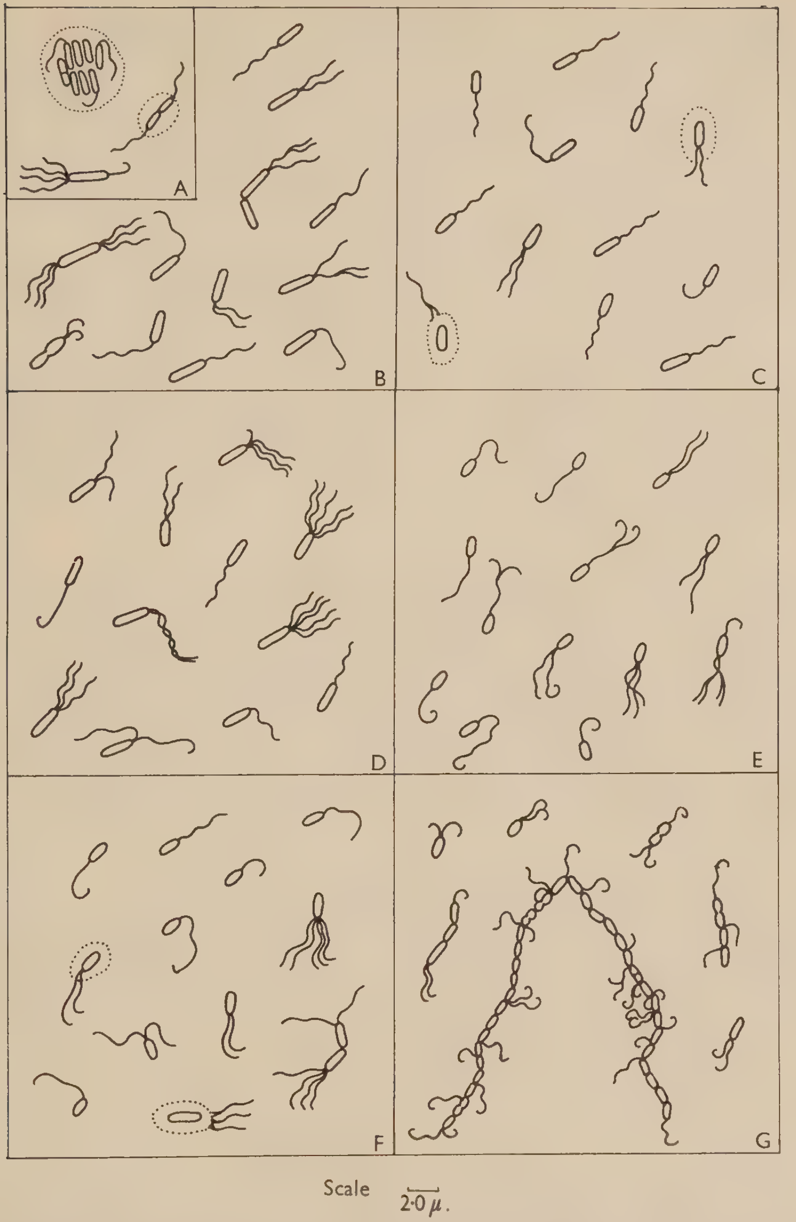

Fig. 1. Depicting the appearance of silver-plated preparations of six isolates of *Pseudomonas* spp. after 24 hr. incubation, and one of these isolates after 10 hr. incubation. (The dotted outline indicates the boundary of the capsule.) A. From 10 hr. culture of isolate 25/1: note polar flagella only, variable number of flagella/rod, communal rods in capsules, one rod with cross septum, variable flagella curvature. B. From 24 hr. culture of isolate 25/1: note polar flagella, variable number of flagella/rod, bipolar flagellation, variable flagella curvature. C. From 24 hr. culture of isolate 24/1, *P. aeruginosa*: note tendency to monotrichy, slightly variable flagella curvature, some rods capsulated, flagella arising from the rod and passing through the capsule. D. From 24 hr. culture of isolate 13/1: note features as for B. E. From 24 hr. culture of isolate 15/1: note features as for B. F. From 24 hr. culture of isolate 16/2: note features as for B. G. From 24 hr. culture of isolate 22/1: note chain formation and apparent lateral flagellation.

body of the organism was overlaying flagella which originated at a pole. The silver-plating technique also revealed that bicellular organisms with a transverse septum, and longitudinal pairs or chains of organisms, were not uncommon. Each non-septate unit or member of a chain was typically polarly flagellate (see Fig. 1, A, G). In small chains or pairs this may easily be mistaken for lateral flagellation if the staining methods used are not sufficiently refined to show individual cell outlines.

Bipolar flagellation was also noticed fairly frequently (see Fig. 1, A, B); in a total of 443 preparations, 119 showed some organisms with flagella at both poles. This feature was not constant for a given isolate, and it was noticed that it was typically confined to rods of greater than average length, or to rods with distinct signs of a central constriction or septum (see Fig. 1, A, B and F). Bipolar flagellation thus seems to be a prelude to division, and the listing in *Bergey's Manual* (1948) of 12 species of *Pseudomonas* as bipolarly flagellate probably has no value in classification, thus supporting the conclusion of Leifson (1951) and Sneath (1956*b*).

Flagella wavelength and amplitude

From the examination of many flagellate organisms in each stained preparation, it was evident that the flagella wavelength and amplitude were often not constant even in a single microscope field selected at random. Figure 1 pictures faithful records of flagella observed in a single stained preparation of each of six isolates. It may be seen that a tendency towards a constant flagella spiral was apparent, but variants were common (see also Pl. 1). Because of this visually obvious variability no detailed measurements or analyses were made.

Flagellation of young cultures

In cultures 0–18 hr.-old convincing lateral flagellation was never observed except in association with chain formation or multicellularity. Only rarely were any flagella visible until after 9 hr. of incubation, and neither the shape nor position of the flagella of young organisms were especially characteristic. Conspicuous slime and/or capsule formation was however often seen in cultures at *c*. 10 hr. and groups of 2–12 flagellate and non-flagellate rods enclosed in a common capsule were frequently observed (see Fig. 1, A).

Slime and capsule formation

In young and in older (18–24 hr.) cultures, conspicuous slime formation was often a striking feature of many of these stained preparations, and unless very dilute suspensions were used the whole background of the film after silver plating was dense black; rods were then seen only in a few places where clear zones were apparent round them (see Pl. 1, fig. 2). These clear zones were at first mistaken for capsules; it is possible that they formed as a result of the drying and contraction of hydrophilic colloidal slime away from the organisms. The degree of slime formation varied from this extreme to very slight, but it was very rare for any of the isolates examined to produce no slime at all. It was once thought that the stained background was silver-plated mordant, because the application of Klieneberger-Nobel's (1950) technique for staining amorphous slime gave negative results. However, it was noted that one slide which contained preparations from three different isolates simultaneously stained, did not show similar backgrounds, so that an intrinsic difference in the distilled-water suspensions from which the respective smears had been made appeared to be probable. Confirmation that the background material in the silver-plated preparations was indeed amorphous slime was obtained by using the Alcian Blue slime-staining method described by Novelli (1953) and by McKinney (1953) who investigated the polysaccharide specificity of this staining reaction. The results obtained in the present work by using the silver-plating and the Alcian Blue staining techniques were in good agreement.

Distinct and definite zones of darker- or lighter-staining material which surrounded the rods in some of the preparations stained by the silver-plating technique strongly suggested the presence of capsules (see Pl. 1, fig. 3). However, only some organisms in any given preparation showed this feature; the width of the capsular zone was not constant in a given field (see Pl. 1, fig. 3). To confirm that these structures were not artefacts, several methods (Anthony, 1931, modified by Tyler, see *Manual of Methods*, 1954; Klieneberger-Nobel, 1948, 1950; Leifson, 1951; Kulka & Walker, 1948; Novelli, 1953) for demonstrating capsules were compared, using some mucoid strains of *Pseudomonas* as test material. In all cases distinct capsulation was shown only by some individual organisms in each preparation; this character did not appear to be very constant or stable and the width of the capsule when seen was also variable.

Protrusion of a flagellum through the capsule was frequently observed (see Pl. 1, fig. 3; Fig. 1, A, C and F). When it was not possible to focus flagella through a capsule, they could be distinguished from fine strands of slime or capsular material which were frequently seen (apparently drawn out from any zone of the capsule) by: (*a*) the uniform diameter; (*b*) the regular spiral shape (usually); (*c*) the darker colour; (*d*) the polar position. The slime strands are not considered to lend any support to Pijper's (1946) theory of bacterial locomotion.

DISCUSSION

The flagella-staining method described here may be recommended for the routine examination of *Pseudomonas* spp. for several reasons, including: no elaborate apparatus or uncommon chemicals are required; the preparations show several cytological features simultaneously, viz. number and position of flagella, chain formation, internal cellular differentiation, slime and capsule formation. During the work reported, comparisons with other flagella-staining methods, e.g. those of Gray (1926) and Leifson (1951), were made, but it was found that these gave a much less refined picture than did the method here advocated. With the former methods the background was usually darker and more granular. Also, individual flagella were not so clearly distinguishable, particularly when more than two were present (as was the case with the typically lophotrichate pseudomonads).

The exact position of the flagella is considered by many to be useful in bacterial classification. Many thousands of flagellate pseudomonads were observed in the stained preparations described in the present work, and lateral flagella were rarely seen on non-septate rods, although flagella probably partly obliterated by the dense body of the organism and thus apparently emerging laterally, were occasionally seen. In my experience, lateral flagella were associated with rod constrictions, cross-septa or chain formation. Electron micrographs of authentic *Pseudomonas* spp. (van Iterson, 1947; Bartholomew 1949; Houwink & van Iterson, 1950; Bisset & Hale, 1951; Bisset 1951) support this opinion. To my knowledge only Bartholomew (1949) has asserted lateral flagellation in *Pseudomonas*; after careful examination of his pictures the present writer feels that it is probable that the true origins of the rarely seen lateral flagella were concealed by the dense bodies of the organisms. However, organisms from single pure cultures with polar or lateral flagella, or both, have now been described by several workers: Leifson & Hugh (1953) for *Aeromonas*, *Alcaligenes* and certain yellow bacteria; Leifson & Palen (1955) for *Listeria*; Sneath (1956*b*) for *Chromobacterium*; Conn & Elrod (1947) for *Rhizobium*. Sneath (1956*b*) reported an investigation of various environmental factors which might affect flagella location. He found that laterally-flagellate chromobacteria were rare in a liquid medium, that organisms from young cultures frequently had many lateral flagella, but that in older cultures only polar flagella were evident. Sneath's observations strengthen the warning of Leifson & Hugh (1953) that lateral flagella in the early stages of growth of many bacteria, e.g. *Aeromonas* (ex *Pseudomonas*), must be recognized. Leifson & Hugh suggested that such young peritrichously-flagellate bacteria were multicellular, and that as growth proceeded polarly-flagellate unicells became dominant. Sneath (1956*b*) rejected this hypothesis after failing to demonstrate more nuclear structures and cross-walls in the peritrichously-flagellate organisms than in the older polarly-flagellate forms. Such observations emphasize the need for a statement of the growth conditions, especially noting the factors known to affect cell division, if the location of bacterial flagella is to be used in classification. The uniform conditions maintained in the present work may account for the consistent polar flagellation observed. The observations described in the present account, including studies of young organisms, confirm the definition of *Pseudomonas* as a polarly-flagellate genus, but do not support further subdivision on the basis of monotrichous or lophotrichous flagellation (as advocated by Leifson, 1951).

Another detail which has been suggested as worthy of consideration for classification purposes is that of the degree of curvature (wavelength and amplitude) of the flagella (Leifson, 1951; Leifson & Hugh, 1953; Leifson & Palen, 1955; Leifson, Carhart & Fulton (1955). From the results of the present work it is concluded that in *Pseudomonas*, even under the reasonably standardized conditions used here, differences in flagella wavelength and amplitude are not sufficiently constant to be a useful character for subgeneric classification. This conclusion may be criticized, as indeed the suggestions of Leifson and his colleagues have been criticized (Pijper & Abraham, 1954;

Pijper, Neser & Abraham, 1956), on the grounds that stained pictures of flagella are artefacts because flagella wavelength is affected by varying environmental factors (e.g. temperature, pH value, colloid content of the medium, drying), which are not constant during the flagella-staining procedure. Much of this criticism is perhaps not valid in the light of Weibull's markedly artefact-producing flagella precipitation experiments, in which pure flagella suspensions or solutions were salted out with ammonium sulphate; in the case of *Proteus*, the resultant spirals had the same period as the flagella on the living organism (Weibull, 1951). From measurements made on living organisms, Pijper *et al.* (1956) recorded that at least two wavelengths were found in *Pseudomonas*; *c.* 1·53 μ. for *P. aeruginosa*, 1·32 μ. for a *Pseudomonas* sp., and 0·60 μ. for *P. diminuta*. This latter appears a striking difference, but the present writer feels that the creation of this new species of *Pseudomonas* by Leifson & Hugh (1954) on the sole basis of the unusual flagella wavelength may be questioned. Indeed the allocation of Leifson & Hugh's organism to the genus *Pseudomonas* may be queried because of lack of pigment, no acid production from glucose, rapid production of acid (which acid?) from ethanol and the high ethanol tolerance; the genera *Vibro* and *Acetobacter* would also seem to qualify for consideration. The desirability of a critical assessment of all known diagnostic characters, together with some comparison with the most nearly related of the numerous *Pseudomonas* species already described, before the creation of further new species of this genus, has already been adequately emphasized by van Niel & Allen (1952).

The author wishes to thank Dr W. J. Dowson for the gift of nineteen cultures of phytopathogenic *Pseudomonas* spp., the Curator of the National Collection of Type Cultures for five named cultures and Imperial Chemical Industries Ltd. (Manchester) for a generous sample of Alcian Blue 8GN.150. The photomicrographs were kindly produced by Mr R. A. Barker; to him, and to Professor B. C. J. G. Knight and Dr T. Gibson for their help in the production of this paper, grateful acknowledgement is expressed.

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EXPLANATION OF PLATE

Pseudomonas spp.; silver-plated preparations of various isolates.

Fig. 1. Isolate 78/2. From 24 hr. culture. Note polarly flagellate rods, different numbers of flagella/organism, occasional apparently lateral flagellation and the tendency toward a uniform flagella curvature. × 2500.

Fig. 2. Isolate 74. From 24 hr. culture. The dense background is due to abundant exocellular slime. Reasons for the clear zones round the rods are not known, but they are not to be mistaken for capsules. Note flagella. × 2500.

Fig. 3. Isolate 42/3. From 24 hr. culture. Note polar flagellation, different numbers of flagella/organism (1–10), amphitrichate rods of larger than average size or showing signs of fission, flagella arising from the rod and passing through the capsule, variable width of capsules, edge of film showing stained exocellular slime, and the slightly variable flagella curvature. × 2500.

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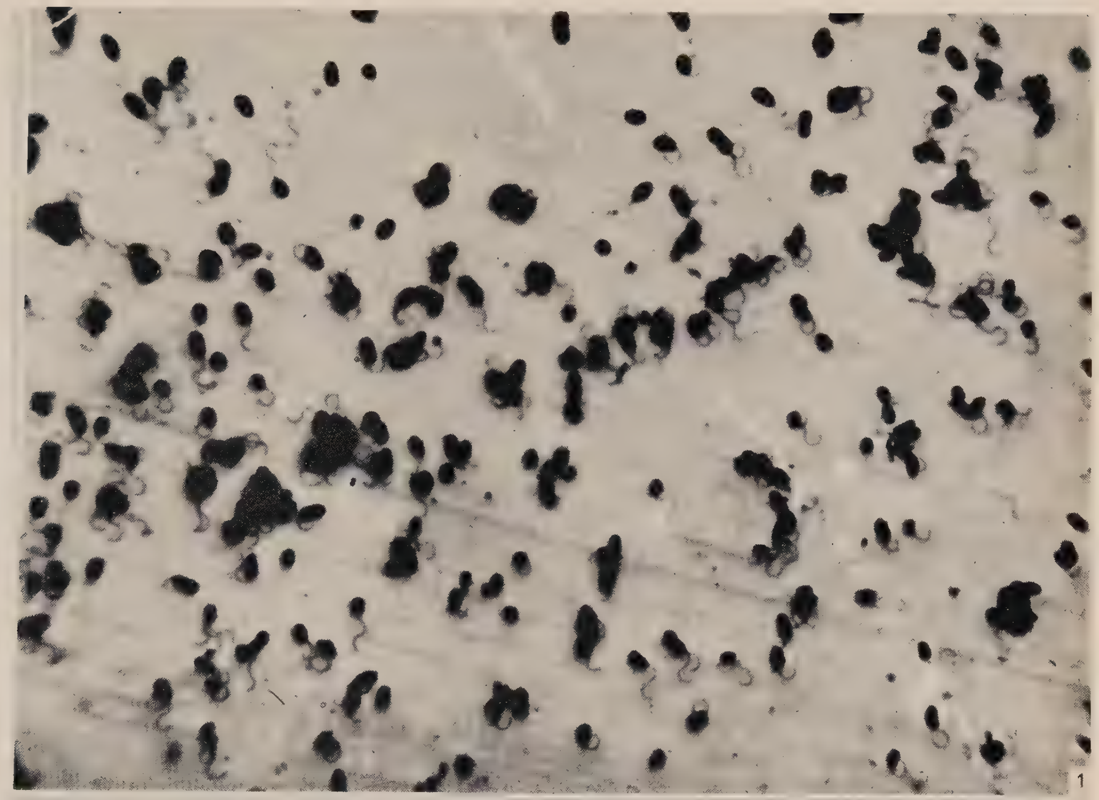

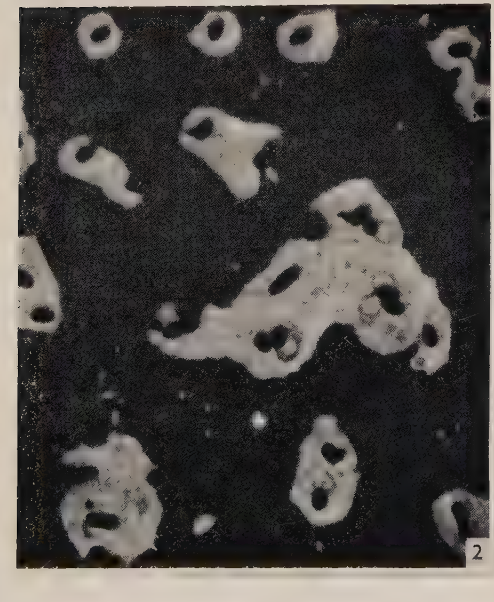

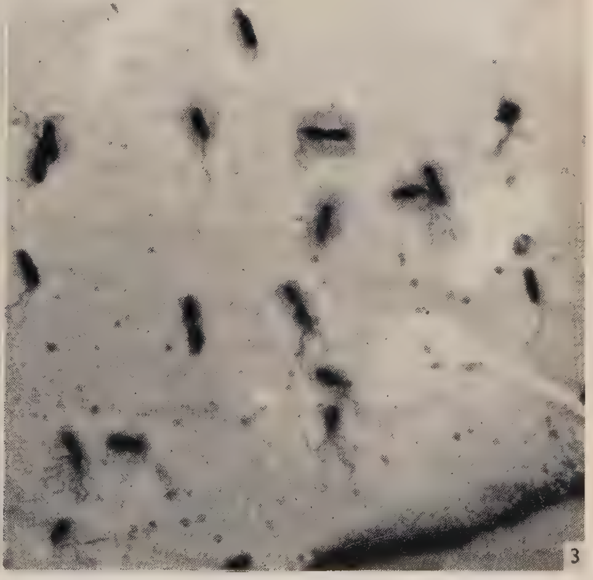

M. E. RHODES—THE CYTOLOGY OF PSEUDOMONAS. PLATE 1

(*Facing p.* 648)

VANĚK, Z., DOLEŽILOVÁ, L. & ŘEHÁČEK, Z. (1958). *J. gen. Microbiol.* 18, 649–657

Formation of a Mixture of Antibiotic Substances including Antibiotics of a Polyene Character, by Strains of Actinomycetes Freshly Isolated from Soil Samples

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SUMMARY: Of a total of 739 actinomycetes freshly isolated from soil samples from China, 515 (69·7 %) were antibiotically active. Their antibiotic spectrum was determined against 14 test micro-organisms, including bacteria, yeasts and fungi, and 386 of them were characterized by paper chromatography using agar blocks, by behaviour on agar plates incorporating ion-exchange resins and by u.v. absorption spectrum (to detect polyene substances). Of these strains, 195 (50·5 %) produced a mixture of antibiotics of both polyene and non-polyene type. On the basis of these results the active strains have been classified in various ways.

Most early studies of the incidence of antibiotic properties in actinomycetes were concerned with antibacterial action (Nakhimovskaya, 1937; Krasilnikov & Koreniako, 1939; Waksman, Horning, Welsch & Woodruff, 1942; Welsch, 1942; Aiso, Arai, Yanagisawa & Nakajima, 1949; Rouatt, Lechevalier & Waksman, 1951), and only occasionally showed an interest in antifungal action (Alexopoulos, Arnott & McIntosh, 1938, 1941; Alexopoulos & Herrick, 1942; Meredith, 1944). In recent years greater interest has been evinced in the study of antifungal polyene antibiotics, which are distinguished by a characteristic u.v. absorption spectrum (Utahara, Okami, Nakamura & Umezawa, 1954; Vining, Taber & Gregory, 1955; Pledger & Lechevalier, 1956; Ball, Bessel & Mortimer, 1957).

It was found early on that the production of antibiotic substances by actinomycetes was not species specific. Under different conditions, a number of stains could produce not one, but several different antibiotics (Perlman, 1953). The ability of actinomycetes to produce a number of antibiotics simultaneously was originally regarded as exceptional, but during the past few years several reports have appeared on the production of mixture of antibiotics by actinomycetes in submerged fermentation, chiefly from Japanese laboratories (e.g. Aiso, 1950; Hosoya, Komatsu & Sonoda, 1951; Takahashi, 1952, 1953; Kikuchi, 1955; Maeda *el al.* 1956; Nakazawa & Shibata, 1953; Ueda & Umezawa, 1956. The present authors concentrated primarily on screening actinomycetes producing a mixture of antibacterial and antifungal substances.

METHODS

Experimental procedure. Strains of actinomycetes were isolated from samples of Chinese soil by a centrifugation method (Řeháček, 1956). This method involved 20 min. centrifugation of a suspension of a sample of the soil at a relative centrifugal strength of 904 *g* at the surface and 1609 *g* at the bottom and thus removed the micro-organisms accompanying the actinomyces contained in the sample of the soil and excluded their influence on the culturing plates. The centrifugation method made it possible to obtain within 10 days colonies of pure cultures of actinomycetes, which could be re-inoculated direct from the first culture medium and might be used for further study. In this way the tedious purification of strains was done away with, so that influence on the isolated actinomycetes by culturing under laboratory conditions was minimal as compared with present isolation methods.

The isolated strains were grown on solid medium in Petri dishes for 5–8 days. Agar blocks were cut and used for determining the antibiotic spectrum, for characterizing by paper chromatography and for determining the ionic character of the antibiotic substances secreted. The rest of the medium was extracted and tested for the presence of polyenes by u.v. absorption. The visual appearance of the pigment formation was recorded. Thus it was possible to study the production and basic properties of antibiotic substances from a single Petri dish culture.

Media and cultivation. The actinomycetes were maintained on glucose potato agar slopes. Petri dish cultures, each 12 cm. dish, contained 30 ml. medium of the following composition (%, w/v): glucose 1, starch 1·5, corn steep 0·5 (dry weight), $(NH_4)_2SO_4$ 0·35, NaCl 0·5, $CaCO_3$ 0·5, agar 2 (Ševčík & Podojil, 1957; Ševčík, Podojil & Vrtišková, 1957), selected as the most satisfactory from among a large number of media tested. The organism was evenly inoculated over the whole surface of the medium.

Test bacteria were maintained on meat-infusion peptone agar, but for determining the antibiotic spectrum, they were grown at 32° on a nutrient medium containing (%, w/v): peptone 0·5, yeast autolysate 0·32 and agar 1·5.

Test yeasts and fungi were maintained, and cultured during the experiment, at 28° on a medium containing (%, w/v): malt extract 0·6, yeast extract 0·3, glucose 1, peptone 0·5 and agar 1·5.

Determination of the antibiotic spectrum. The authors used the agar block method of Ishida, Shiratori, Okamoto & Miyazaki (1951). Agar blocks 8 mm. in diameter were cut from Petri dish cultures and placed in wells in an inoculated agar medium in glass dishes measuring 35 cm. × 35 cm. One dish held 100 blocks. Incubation was for 20 hr. except that with *Alternaria solani* and *Fusarium nivale*, when 48 hr. was used. After incubation the inhibition zones were measured.

The following test micro-organisms were used: *Bacillus subtilis*, *Staphylococcus aureus*, *Sarcina lutea*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Candida albicans*, *Saccharomyces cerevisiae*, *Penicillium duclauxi*, *Asperigillus niger*, *Rhizopus nigricans*, *Fusarium nivale*, *Alternaria solani* and *Trichoderma* sp., all from the collection of the Czechoslovak Academy of Science.

Determination of Character of antibiotics. Character of antibiotics was determined by the paper chromatography method elaborated by Ševčík *et al.* (1957). Agar blocks from Petri dish cultures of the actinomycete were placed on strips of chromatographic paper measuring 1 cm. × 18 cm. After allowing

30 min. for diffusion the papers were developed in a system of ten solvents: distilled water, 3% NH_4Cl, 100% methanol, 100% acetone, ethylacetate saturated with water, *n*-butanol saturated with water, diethyl ether saturated with water, chloroform, benzene and light petroleum.

When developed, the strips of chromatographic paper were placed on agar medium in glass plates measuring 35 cm. × 35 cm.

Test micro-organisms suitable for biological investigation were selected on the basis of the antibiotic spectrum, In some actinomycetes, production of a mixture of antibiotics could be deduced from the results of biological investigation of the summarized papergram, from the formation of double inhibition zones and from the very broad antibiotic spectrum. In such cases two or more test micro-organisms were used for the biological investigation of the summarized papergram.

Assay of ionic character. Agar blocks with the culture of actinomycetes were placed in the basic agar medium, over which an agar medium containing cation exchange resin ROA (Severa, Pecák & Hoffman, 1954) or Fluka STG anion exchange resin (A.G. St Gallen) was poured. Agar medium inoculated with a test micro-organism was then poured over the agar medium containing ion exchange resin. The results were evaluated by comparing the averages of inhibition zones obtained after 18 hr. incubation at 37° in the medium containing ion exchange resin and the averages of inhibition zones in the control part of the plate containing no ion exchange resin. This method was elaborated by Ševčík & Podojil (1957).

Detection of substances of a polyene character. The agar medium remaining after the previous tests was ground, washed with acetone and this extract was dried until completely dry. The u.v. absorption spectrum of an ethanolic extract of the residue was then examined.

RESULTS

Effect of the actinomycetes on individual test micro-organisms

Antibiotic activity was detected in 515 (69·9%) of 739 strains of actinomycetes recovered from 115 soil samples. The percentage of strains active against various Gram-positive and Gram-negative bacteria is given in Fig. 1, and against yeasts and fungi in Fig. 2. From Fig. 1 it will be seen that groups C and B contained actinomycetes inhibiting both Gram-positive and Gram-negative bacteria. The percentage of strains inhibiting only Gram-positive bacteria is larger, however. Figure 2 shows that group D, i.e. with inhibition zones of 31–40 mm., contained actinomycetes inhibiting *Saccharomyces cerevisiae*, *Rhizopus nigricans* and *Fusarium nivale*. Group C, with inhibition zones of 21–30 mm., included strains inhibiting all the test micro-organisms with the exception of *Alternaria solani*. Although *Rhizopus nigricans* was inhibited by a large number of weakly active strains (39%), it was inhibited by only a small proportion of actinomycetes with medium activity (3·3%).

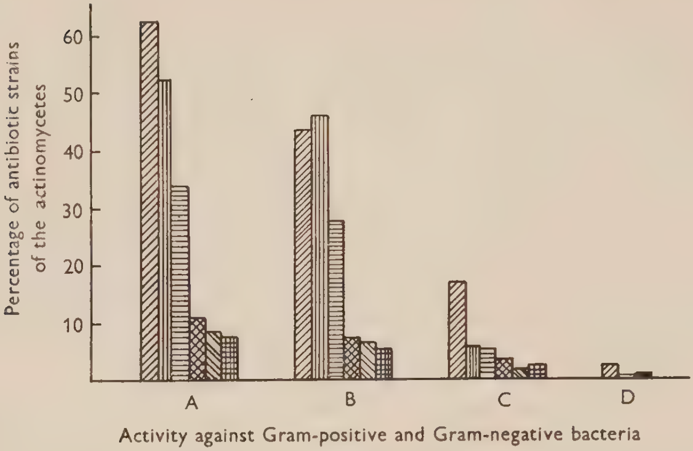

Fig. 1. The percentage of strains of the actinomycetes active against various Gram-positive and Gram-negative bacteria. A: percentage of strains showing activity of any degree; B, percentage of strains showing weak activity, diameter of inhibition zone 10–20 mm.; C, percentage of strains showing moderate activity, diameter of inhibition zone 21–30 mm.; D, percentage of strains showing strong activity, diameter of inhibition zone 31–40 mm. The diameter of the inhibition zone includes the central agar plug of a diameter of 8 mm. ▨, *Sarcina*; ▤, *Staphylococcus aureus*; ▧, *Pseudomonas*; ▥, *Bacillus subtilis*; ▩, *E. coli*; ▦, *Serratia.*

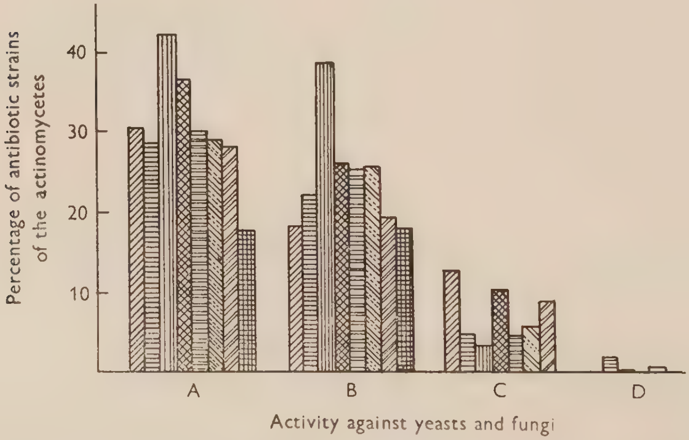

Fig. 2. The percentage of strains of the actinomycetes active against various yeasts and fungi. A, percentage of strains showing activity of any degree; B, percentage of strains showing weak activity, diameter of inhibition zone 10–20 mm.; C, percentage of strains showing moderate activity, diameter of inhibition zone 21–30 mm.; D, percentage of strains showing strong activity, diameter of inhibition zone 31–40 mm. The diameter of the inhibition zone includes the central agar plug of a diameter of 8 mm. ▨, *Candida*; ▥, *Rhizopus*; ▤, *Fusarium*; ▨, *Trichoderma*; ▤, *Saccharomyces*; ▩, *Penicillium*; ▧, *Aspergillus*; ▦, *Alternaria.*

Action of actinomycetes on groups of test micro-organisms

In view of the fact that the recently described group of polyene antibiotics is active against yeasts and fungi, but not against bacteria, and as the number of known antibiotics with an antibacterial and antifungal effect is very small, it can already be inferred from the results of this part of the work, as Fig. 3 shows, that a large number of the actinomycete strains in the present series produces a mixture of antibiotic substances.

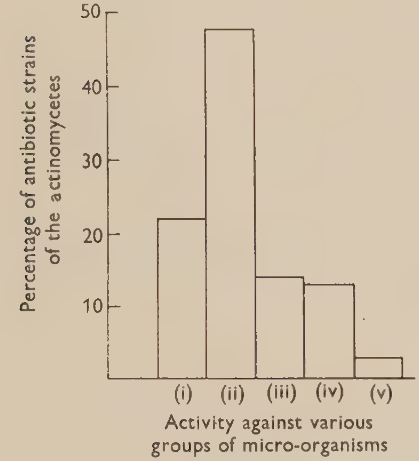

Fig. 3

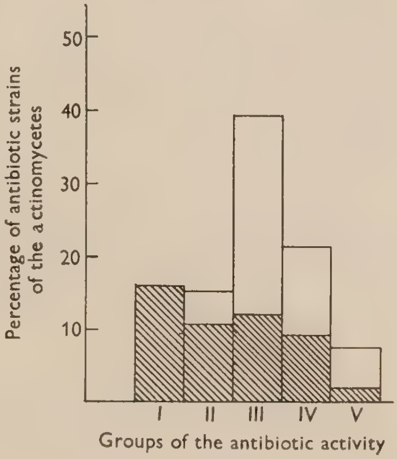

Fig. 4

Fig. 3. **Division of the antibiotic actinomycetes into five main groups, according to their general effect against Gram-positive bacteria (i); against Gram-positive bacteria, yeasts and fungi (ii); against Gram-positive bacteria, Gram-negative bacteria, yeasts and fungi (iii); against yeasts and fungi (iv); and against Gram-positive and Gram-negative bacteria (v).**

Fig. 4. **Classification of the antibiotic activity of 386 of the active actinomycetes into the five main groups, proposed by Waksman & Lechevalier (1953). Shaded parts of columns show a simultaneous production of antibiotics of polyene character.**

Rough classification of antagonistic strains according to the character of the antibiotics and the percentage of strains simultaneously producing antibiotics of polyene character

On the basis of the tests described the authors classified the antibiotic activity of 386 of the active actinomycetes into the five main groups proposed by Waksman & Lechevalier (1953). The results are illustrated in Fig. 4.

Group I

Strains producing actinomycin, xanthomycin and other antibiotics, also pigments of a quinone or anthocyanin character, the colour of which depends on the nature of the substrate (e.g. rhodomycin, thodomycetin, actinorhodine, lithmocidin and ceolicolorin).

The antibiotic substances of this group are soluble in organic solvents but only slightly in water. These strains produce yellow, orange or red pigments, and are active chiefly against Gram-positive bacteria and fungi, and only to a very small degree against Gram-negative bacteria.

Figure 4, col. II, shows that from among the total number of antagonistic actinomycetes, 15·7 % produced antibiotics belonging to this group; 10·6 % produced a mixture of this type of antibiotic together with an antibiotic or antibiotics of a polyene nature.

Group II

Actinomycetes producing streptothricin, streptomycin, neomycin and other similar antibiotics.

The antibiotic substances in this group are of a basic character, easily soluble in water but not soluble in the majority of organic solvents. They have antagonistic effect on Gram-positive and Gram-negative bacteria and also on fungi.

It can be seen from Fig. 4, col. III, that 39·3 % of the total number of antagonistic actinomycetes producing antibiotic substances belonged to this group; 11·9 % produced a mixture of this type of antibiotic together with an antibiotic or antibiotics of a polyene nature.

Group III

Actinomycetes producing antibiotic substances of a neutral or amphoteric character, e.g. chloramphenicol, chlortetracycline, oxytetracycline and flaveolin, and basic substances of the erythromycin-carbomycin type.

The antibiotic substances in this group are weakly soluble in water and easily soluble in organic solvents. Cultures exert antagonistic effect on Gram-positive and Gram-negative bacteria and some are also effective against fungi.

Figure 4, col. IV, shows that of the total number of antagonistic actinomycete strains, 21·4 % produced antibiotic substances belonging to this group. In addition, 9·3 % produced substances of a polyene character.

Group IV

Actinomycetes strongly active against yeasts and less effective against fungi. It can be seen from Fig. 4, col. V, that of the total number of antagonistic actinomycetes, 7·3 % produced antibiotics belonging to this group. At the same time, 1·8 % produced polyene antibiotics.

Group V

Strains of actinomycetes producing substances of a polyene character, with characteristic absorption spectra in u.v. light. These strains exhibit marked antagonistic activity against fungi and are not effective against bacteria.

Fig. 4, col. I, shows that 16·3 % of the total number of actinomycetes produced polyene antibiotics only; 34·2 % produced polyene antibiotics together with antibiotic substances of other groups. Of the total number of 195 strains of actinomycetes which produced antibiotics of a polyene character, 175 produced hepataenes, one strain hexaene, five strains pentaenes, six strains tetraenes, six strains a mixture of heptaenes and pentaenes and two a mixture of hexaenes and tetraenes.

DISCUSSION

In these experiments almost 70% of freshly isolated actinomycetes had an antagonistic effect. In the literature the figures range from 10 to 60%, depending on the methods of testing and the nutrient media. A further factor which influences the number of antagonistic strains detected is marked lability of productive capacity. Thus under laboratory conditions, strains of actinomycetes often lose their ability to produce antibiotic substances after a few subcultures. In the present case this is unlikely to have occurred since all tests were always made from a single Petri dish culture constituting the third subculture after isolation.

A high proportion of freshly isolated strains produce substances of a polyene character as well as other antibiotics. This finding is significant, both from the aspect of methods and from the theoretical point of view. Thus the results of a first screening based on the antibiotic spectrum, solubility and antibiotic properties must be interpreted with great caution, and the possibility that a given strain may produce a mixture of antibiotics must always be taken into account. It should be emphasized that the authors concentrated on the problem of the distribution of strains producing a mixture of non-polyene and polyene antibiotics, which are easily distinguished from one another. The possibility of determining other types of antibiotic mixtures was not investigated.

The results of the work are in agreement with those of Ball *el al.* (1957) in that the majority of antifungal antibiotics found were also of a polyene character. They differ, however, in the proportional distribution of the individual groups. In the findings of Ball *et al.* (1957), the largest group was that of the tetraenes, while in the present experiments the most numerous were the heptaenes, in agreement with Pledger & Lechevalier (1956).

From the incidence of mixture of antibiotics in the individual groups, it is seen that the strains producing polyenes as well as other antibiotics are most numerous in the second group (actinomycins, rhodomycins, rhodomycetin, lithmocidin, etc.) and in the fourth group (antibiotics of the type of tetracycline and erythromycin-carbomycin). It is assumed that this is not a chance phenomenon and that it indicates a definite biogenetic relationship between these antibiotics.

Actinomycetes producing a mixture of antibiotic substances with an antifungal and an antibacterial action are doubtless better equipped for the struggle for existence under the complex competitive conditions which exist among soil microflora. It may be presumed that the formation of mixtures of antibiotic substances is a regular phenomen caused by the interaction of soil microflora and that antibiotic substances are not a chance pathological products of microbial metabolism.

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Cell Death and 'Unbalanced Growth' in Neurospora

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SUMMARY: Mutant spores of *Neurospora crassa* incubated on minimal medium showed two main types of behaviour. One mutant type survived incubation for a week on minimal medium and then responded to the addition of growth substance. The second mutant type died after incubation for one or two days. Strains which died were characterized by an ability to germinate in minimal medium and to begin immediately synthetic processes such as the synthesis of protein and nucleic acid. Addition of ethionine to minimal medium prolonged the survival of strains which committed 'suicide'. Non-germinated Neurospora conidia withstood freezing and storage at −10° for extended periods, whereas conidia which had been incubated in growth medium died, indicating a difference between dormant and synthetically active spores in their response to the environment. The suicide phenomenon is reminiscent of the cell death due to 'unbalanced growth' reported for the thymine-requiring mutant of *Escherichia coli*. In this case, however, cells requiring substances other than nucleic acid constituents display the effect.

Mutant strains of the ascomycete *Neurospora crassa* can be divided into two major types when conidiospores are incubated in a minimal medium. The first type will survive for relatively long periods on an agar plate containing minimal medium and will then respond to the addition of its growth requirement by the production of visible colonies. The second type dies after a short incubation period; i.e. does not respond to addition of growth factor. This behaviour was first observed by Stevens & Mylroie (1953) during studies on the validity of the 'back-mutation' test in Neurospora, but as yet there have been no studies of the cause of the phenomenon nor on the difference between the two classes of mutants. A very similar phenomenon was described by Fries (1948) in Ophiostoma and by Macdonald & Pontecorvo (1953) in *Aspergillus nidulans*. Macdonald & Pontecorvo showed that spores of a biotin-requiring mutant died off rapidly in a biotin-deficient medium. The very interesting observation was made that in certain cases the introduction of a second mutation drastically increased survival in minimal medium, e.g. with biotin- and adenine-requiring or with biotin- and sulphite-requiring strains. Cohen & Barner (1954) described a thymine-requiring mutant of *Escherichia coli* which committed 'suicide' when incubated in a medium devoid of thymine. These authors attributed death of the mutant to unbalanced growth since in the absence of thymine quantities of ribonucleic acid (RNA) and protein were formed but deoxyribonucleic acid (DNA) was not. The organisms swelled and increased in mass but did not divide. Eventually, if allowed to continue to synthesize in a medium devoid of thymine, mutant organisms lost their ability to respond to thymine upon its addition. The cause of 'death' was supposedly due to imbalance in the ratio: (RNA + protein): DNA.

It is evident that organisms which have lost the ability to synthesize single essential constituents, i.e. biochemical mutants, must perforce carry out unbalanced synthesis when they are incubated in a medium devoid of their growth requirement. Initially, at least, all the synthetic processes which do not involve the missing constituent may proceed, whereas those involving the requirement will not. This condition is, formally at least, one of imbalance. We have therefore attempted to answer two closely related questions: (1) is the 'suicide' of mutant conidia incubated on minimal medium related to their unbalanced synthesis; (2) why do certain mutants survive for long periods on a growth medium lacking only one constituent? As as result of these investigations we are convinced that the suicide effect is due to the initiation of synthetic processes in certain mutant types. Secondly, it appears that starvation death only occurs in spores that have been interrupted in synthetic processes which they have already started. Those mutant spores which survive on minimal medium for long periods do not break the (synthetically) dormant state of the conidiospore and are therefore not susceptible to starvation death.

METHODS

In most of the previous investigations with Neurospora, conidia were collected from cultures grown on solid medium after growth periods ranging from 4 to 8 days (Strauss, 1956). Spores are formed throughout the growth period and what is actually obtained is a population of spores of different ages. In order to compare the behaviour of spores of different strains we developed a method for the production of spores differing in their ages (from time of formation) by not more than a few hours. This method is based on a method of Zalokar (1954) for the study of carotenoid formation in Neurospora. Mycelial pads are obtained by permitting growth to occur in a 250 ml. Erlenmeyer flask for 3 days at 30° in 50 ml. minimal medium (Beadle & Tatum, 1945) containing 0·23% (w/v) sodium acetate (tri-hydrate) + two drops of 'Tween 80' to inhibit the formation of aerial hyphae. At the conclusion of the growth period the pads are harvested on a Buchner funnel, washed thoroughly with sterile distilled water and transferred to Petri dishes containing filter paper. The semi-dry pads are then soaked by the addition of 4 ml. 0·1 M-phosphate buffer (pH 6·0) and allowed to incubate at 25°. There is no spore formation until after 12–13 hr. (Fig. 1), after which formation of conidia begins and continues to a maximum reached after 15–16 hr. Conidiation of the mutants used (with the exception of the *at suc* strain) occurs as well in the absence of growth factor as in its presence. It is likely, however, that new protein synthesis is required for conidiation and that the mutants obtain their growth factor from endogenous sources. Ethionine inhibits conidiation and this inhibition is overcome in a competitive fashion by methionine (Table 1). Ammonium ion inhibits conidiation by the *at suc* strain and this inhibition is overcome by succinate as is ammonium inhibition of the growth process (Strauss, 1956). Inhibition by ethionine is effective only when the ethionine is added within the first 9 hr. of incubation. Since the conidia are formed on aerial hyphae we suspect that the 'latent period' before conidiation represents the time required for the aerial hyphae to form. The growth process in this conidiation system might well be the production of these hyphae.

Table 1. *Interaction of* DL-*ethionine and* L-*methionine on conidiation in* Neurospora crassa

Conidiation is recorded as the optical density at 625 mμ. of the conidial suspension obtained by collecting the conidia produced by one pad, filtering through gauze and then making the suspension to 50 ml. Incubation for 16 hr. at 25°. Additions in 0·1 M-phosphate buffer (pH 6·0). Total amount of buffer added in all cases = 4·0 ml.

Concentration of L-methionine (μM)	Concentration of DL-ethionine added (μM)			
	0	0·5	1·0	1·5
	Degree of conidiation			
0	0·28	0·21	0·08	—
	0·32	0·24	0·17	—
	0·35	—	—	—
2	0·27	0·19	0·06	0·05
	0·20	0·24	0·08	0·07
5	0·20	0·16	0·19	0·15
	0·22	0·22	0·18	0·09
10	0·18	0·10	0·23	0·23
	0·14	0·205	0·28	0·24

In the following experiments conidia were prepared by the method indicated. Mutants were grown by the addition of the appropriate growth factor to minimal medium with acetate and 'Tween 80'. Washed mycelial pads were then allowed to incubate in Petri dishes for 17–18 hr. The conidia were collected by washing the surface of the mycelial pad with water and filtering through gauze to remove mycelial fragments. After collection the conidia were washed twice with sterile water by centrifugation. Incubation of conidia in our experiments was generally at 30° except where otherwise indicated. When incubation was in liquid medium, a digestion tube containing a suspension of conidia (35 ml.) was immersed in a water bath and kept agitated and aerated by the passage of a current of air.

In the course of these experiments we attempted to measure viability, germination, nuclear division, protein synthesis, total nucleic acid and oxygen consumption. Viability is defined in these experiments as the ability of a conidium to form a visible colony when plated on an agar medium suitably supplemented with growth factor and containing 1% (w/v) sorbose + 0·1% (w/v) sucrose (Tatum, Barratt & Cutter, 1949). Germination is reported as percentage conidiospores with a definite germ tube emerging from the spore. Nuclear division was determined by comparison of the average nuclear count of conidia before and after varying periods of incubation. A modification of the method of Huebschman (1952) described by Zalokar (personal communication) was used. In this method 'fixed' conidia are stained with azure A after 15 min. hydrolysis with N-HCl at 55°. After two sulphite washes the conidial suspension is dehydrated by passing through ethanol and mounted. The whole staining procedure is carried out in a centrifuge tube, the conidial suspension being placed on a slide only after the final dehydration. While the Feulgen stain has been successfully used we find, in agreement with Zalokar, that the nuclei stain more clearly with azure A. Total protein synthesis was determined as the amount of $^{35}SO_4$ incorporated into the fraction of conidia remaining after extraction with cold 10% (w/v) trichloroacetic acid, 80% (v/v) ethanol in water, a mixture of ethanol (3 vol.) + diethyl ether (1 vol.) (4 times at 60°) and 0·5 N-perchloric acid (4 times at 70°). Chromatography of this residual fraction after hydrolysis with 6 N-HCl indicated that sulphur was present only as cyst(e)ine and methionine. Total nucleic acid was determined on the perchloric acid extract mentioned above. Nucleic acid was taken as proportional to the optical density of the extract at 260 mμ. minus the optical density at 290 mμ., as measured in the Beckman spectrophotometer. The absorption spectrum of the perchloric acid extract indicated a peak at about 260 mμ. Oxygen consumption was determined by standard manometric techniques.

Organisms. The following strains of *Neurospora crassa* were used in these investigations. Strain 74A, a wild-type, and the following amino acid-requiring strains: 584 (methionine), 684 (leucine), 782 (lysine) and 830 (arginine), all obtained from Dr Adrian Srb (Cornell University). The four mutants were derived from the 74A wild-type. Strain 37401, an inositol-requiring strain, and strain 37803, a vitamin B_6-requiring strain, were obtained from Mrs Mary Mitchell (California Institute of Technology). Strains *ad* 1 and *ad* F12, both adenine-requiring, were obtained from Dr Norman Giles (Yale University). The *at suc* strain is the modified succinate-requiring strain previously described (Strauss, 1956). The growth-response curves for four of these strains are given in Fig. 2. The strains used cover a range of growth requirements of about a thousandfold; the requirement of the vitamin B_6-requiring strain being satisfied by about 2 μg./20 ml., whereas the amino acid-requiring strains require, typically, about 2 mg./20 ml. medium. Growth has been determined as the dry weight of mycelium produced after 3 days by an inoculum of spores into 20 ml. medium contained in a 125 ml. Erlenmeyer flask and incubated at 25°.

RESULTS

The inositol-requiring, vitamin B_6-requiring and succinate-requiring (*at suc*) strains started to die rapidly after 1–2 days of incubation on minimal medium (Fig. 3); the leucine-, lysine- and methionine-requiring mutants showed only slight changes in viability even after incubation for 7 days, while the adenine- and arginine-requiring mutants were intermediate. The fraction of these latter two strains which survived incubation for 7 days was *c.* 0·3. In these studies mutant conidia were spread on minimal sorbose plates and then, after the desired time interval, a sufficient quantity of growth factor was injected under the surface of the agar by a hypodermic syringe and allowed to diffuse up through the agar. When conidia of the inositol-requiring strain, 37401, were incubated overnight with shaking in liquid minimal medium they lost their ability to take up oxygen even when inositol was added (Table 2) and even though the incubated material had increased in dry weight as compared with non-incubated controls. At the conclusion of the incubation period the inactivated inositol-requiring conidia formed small clumps.

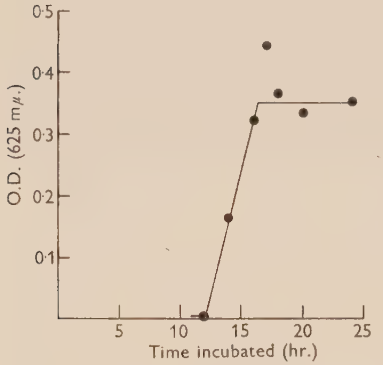

Fig. 1

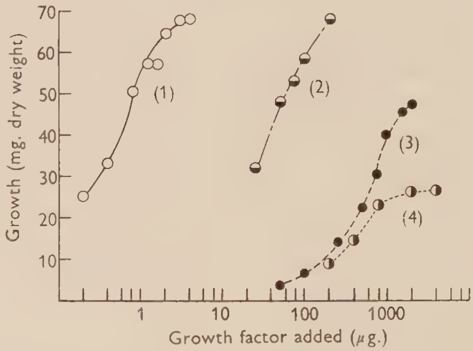

Fig. 2

Fig. 1. Conidiation as a function of incubation time. Conidiation is recorded as the optical density at 625 mμ. of the conidial suspension obtained by collecting the conidia produced by one pad, filtering through gauze to remove mycelial fragments and then making the suspension to 35 ml. Incubation was at 25° with 4 ml. of 0·1 M-phosphate buffer (pH 6·0). Values are the average of duplicates.

Fig. 2. Response of mutants of *Neurospora crassa* to their required growth factor. The amount of growth factor indicated was added to 20 ml. minimal medium. Growth is recorded as the mg. dry weight mycelium produced after incubation for 3 days at 25° in static culture. Curve (1): mutant 37803, vitamin B_6-requiring; curve (2): mutant 37401, inositol-requiring; curve (3): mutant *ad* F 12, adenine-requiring; curve (4): mutant 684, leucine-requiring.

Table 2. *Loss of the ability of the inositol-requiring mutant* (37401) *of* Neurospora crassa *to respire after incubation in minimal medium*

Equal volumes of a conidial supension of mutant 37401 were added to a series of flasks containing 50 ml. minimal medium. Refrigerated set kept 24 hr. standing in cold. Incubated-shaken 24 hr. at 28°. Conidia were collected and washed twice, and one set was used for dry weight. Material from the other flasks was pooled and made to 10 ml. with minimal medium. 2 ml. of the resulting suspension was added to manometers and the oxygen consumption was determined at 30°.

Condition	Dry wt. after experimental period (mg.)	Oxygen uptake (1 hr.; μl.)	
		Minimal medium	Minimal + inositol
Refrigerated	10·1	171	163
		179	199
Incubated at 28°	23·4	7	15
		0	10

The inositol- and vitamin B_6-requiring strains germinated readily in minimal medium (Fig. 4), the amino acid-requiring mutants did not germinate at all after aeration for 6 hr. in minimal medium, while the adenine-requiring strains showed very slight germination. We used a conservative estimation in these experiments; a spore was scored as germinated only when an unambiguous outgrowth was seen. In most of the following experiments we arbitrarily used the inositol-requiring strain as typical of those strains which kill themselves, while the leucine-requiring strain was selected as typical of strains which survived.

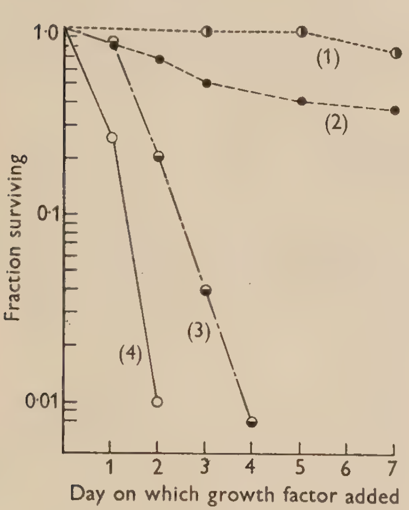

Fig. 3

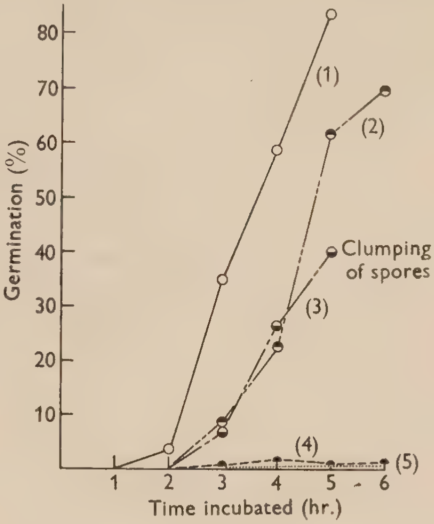

Fig. 4

Fig. 3. Survival of mutants of *Neurospora crassa* incubated on minimal medium. Growth factor was injected under the surface of the agar after incubation at 25° for the time indicated. The plates were then incubated an additional 3–4 days. Curve (1): leucine-requiring; curve (2): adenine-requiring; curve (3): inositol-requiring; curve (4): vitamin B_6-requiring.

Fig. 4. Germination of mutant and wild-type strains of *Neurospora crassa*. Conidia were incubated with aeration in 35 ml. minimal medium at 30° for the times indicated. Germination was determined by microscopic count of formalin-fixed material; at least 500 conidia were scored for each point. Curve (1): vitamin B_6-requiring; curve (2): wild-type; curve (3): inositol-requiring; curve (4): adenine-requiring; curve (5): leucine-requiring.

Germination represents true synthesis on the part of the conidia since the formation of an outgrowth was invariably preceded by protein and nucleic acid synthesis (Figs. 5, 6). We have not, as yet, been able to measure the DNA content of Neurospora conidia so that we cannot compare RNA:DNA ratios as was done by Cohen & Barner (1954). It has been possible to show an increase in the average number of nuclei/conidium on incubation of both wild-type and the inositol-requiring strain in minimal medium by the azure A technique and by the increase in the number of Feulgen-staining bodies (Table 3). It is therefore quite likely that the increase in total nucleic acid includes an increase in DNA.

The Neurospora conidium is multinucleate; a population of conidia of strain 74A has a distribution of nuclei/conidium with an average of 2·6. This average may differ from strain to strain and is determined partly by the medium on which the conidiating mycelium is grown (Huebschman, 1952). The average number of nuclei/conidium increases on incubation in minimal medium and has increased to an average of 3·5 after incubation for 2 hr. After incubation for 2 hr. no germination can be discerned. It therefore seems that nuclear division precedes germination in Neurospora.

Table 3. *Distribution of nuclei in* Neurospora conidia

		Duration of incubation (hr.)			
Strain of *N. crassa*		0	1	2	4
Wild-type (74A)	Conidia counted	454	556	566	526
	Mean: nuclei/conidium	2·61	2·76	3·47	5·42
	Mode: nuclei/conidium	2	2	3	5
Inositol-requiring (37401)	Conidia counted	580	592	669	491
	Mean: nuclei/conidium	2·36	2·79	3·29	4·97
	Mode: nuclei/conidium	2	2	3	5
Leucine-requiring (684)	Conidia counted	620	385	419	461
	Mean: nuclei/conidium	2·38	2·56	2·48	2·55
	Mode: nuclei/conidium	2	2	2	2

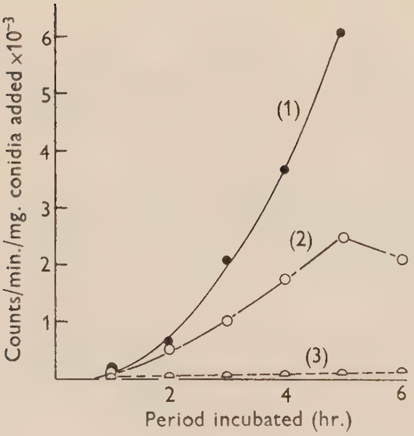

Fig. 5

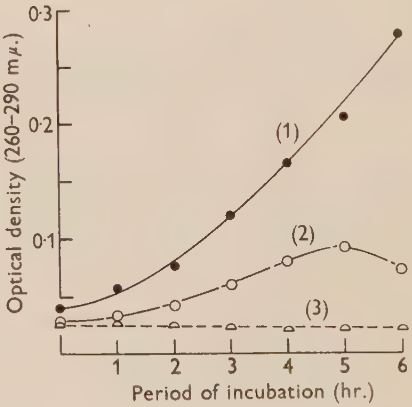

Fig. 6

Fig. 5. Protein synthesis by conidia of *Neurospora crassa*. Conidia were incubated as before in minimal medium containing $^{35}SO_4$. Protein synthesis is measured as the incorporation of ^{35}S into the residual fraction (see text for details). Curve (1): wild-type; curve (2): inositol-requiring; curve (3): leucine-requiring.

Fig. 6. Total nucleic acid synthesis by conidia of *Neurospora crassa*. Conidia were incubated in minimal medium as before. Nucleic acid synthesis was measured as the difference in optical density (260–290 mμ.) of the material extracted with perchloric acid after preliminary cold acid and ether + ethanol extraction (see text for details). Curve (1): wild-type; curve (2): inositol-requiring; curve (3): leucine-requiring.

There was no major difference in the rate at which conidia of the leucine-requiring mutant, which did not commit suicide, took up oxygen when suspended in buffer as compared to wild-type conidia or to conidia of the inositol-requiring strain which did commit suicide (Table 4). Furthermore, conidia of both mutant strains were able to oxidize sucrose both in buffer and in minimal medium at a rate comparable to that of the wild-type. It is difficult to decide whether the increased rate of oxygen uptake in minimal medium compared to sucrose buffer shown by both wild-type and inositol-requiring strains during the first hour of incubation was significant, but at any rate this increase was not shown by conidia of the leucine-requiring strain. It is evident that conidia of both the wild-type and mutant strains oxidized carbohydrate at essentially the same rate. Neither of the two mutant strains can be considered to produce spores which are 'dormant' compared to the wild-type as far as their ability to degrade carbohydrate is concerned.

Table 4. *Oxygen uptake by wild-type and mutant conidia of* Neurospora crassa

Recorded O_2 consumption at 30° per conidium during the first hour after addition. Manometer contents: main compartment—2·0 ml. conidial suspension in 0·1 M-phosphate buffer (pH 6·0); side arm—(*a*) 0·5 ml. buffer, (*b*) 0·5 ml. 10 % sucrose, (*c*) 0·5 ml. of minimal medium at fourfold concentration.

		Addition		
Strain of *N. crassa*	No of conidia added	Buffer	Sucrose	Minimal medium
		Oxygen consumption (μl. O_2/hr./conidium)		
Wild-type (74A)	$1{\cdot}7 \times 10^8$	$3{\cdot}7 \times 10^{-7}$	$24{\cdot}1 \times 10^{-7}$ $22{\cdot}5 \times 10^{-7}$	$27{\cdot}4 \times 10^{-7}$
Inositol-requiring (37401)	$2{\cdot}12 \times 10^8$	$4{\cdot}2 \times 10^{-7}$	$16{\cdot}5 \times 10^{-7}$ $15{\cdot}8 \times 10^{-7}$	$20{\cdot}0 \times 10^{-7}$ $20{\cdot}0 \times 10^{-7}$
Leucine-requiring (684)	$3{\cdot}06 \times 10^8$	$4{\cdot}4 \times 10^{-7}$	$22{\cdot}4 \times 10^{-7}$ $22{\cdot}8 \times 10^{-7}$	$21{\cdot}9 \times 10^{-7}$ $23{\cdot}2 \times 10^{-7}$

We attempted without any positive result to determine whether the inositol- or vitamin B_6-requiring strains incubated in minimal medium produced some soluble product which was either toxic to these strains or to wild-type, or which stimulated germination. Filtrates of minimal medium in which dense suspensions of the inositol- or vitamin B_6-requiring mutants had been incubated overnight with shaking did not affect the germination, ability to form colonies on suitable medium or the dry weight attained by either the wild-type or the mutants when such filtrates were added to appropriate media.

Since it seemed possible that the growth of the mutants placed on minimal plates contributed in some way to their death, the effect of an inhibitor of germination on the survival of mutant conidia was determined. Ethionine was selected as the inhibitor because it readily inhibited germination and because its effects could be counteracted by the injection of methionine into the minimal plate containing ethionine at the same time, when growth factor was added. Ethionine had a very marked effect in prolonging survival (Fig. 7) when small amounts were incorporated into minimal medium. At the ethionine concentration used, growth eventually occurred since wild-type conidia plated on medium containing ethionine at this concentration eventually produced colonies. The phenylalanine analogue, DL-*p*-fluorophenylalanine, also prolonged surival of the vitamin B_6-requiring mutant in a similar experiment. Lowering the temperature of incubation also increased survival. Neurospora conidia retained their viability after being quick frozen and conidia of the inositol-requiring strain kept at −10° for 6 days formed colonies on thawing and the addition of growth factor.

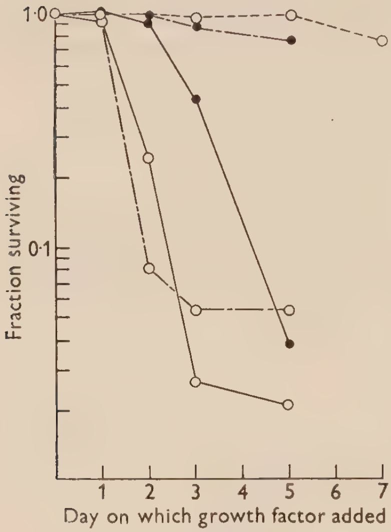

Fig. 7

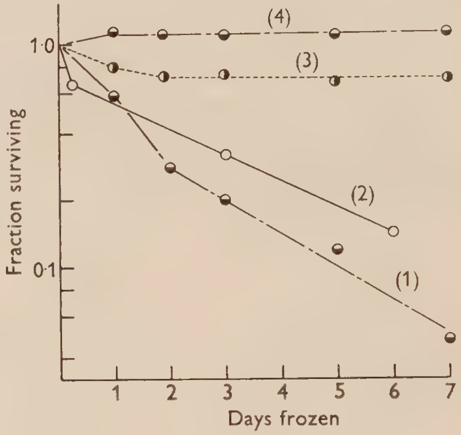

Fig. 8

Fig. 7. Effect of ethionine on the survival of *Neurospora crassa* mutants incubated on minimal medium. Open circles: cultures incubated on minimal medium. Filled circles: cultures incubated on minimal medium (25 ml.) containing 0·2 μmole DL-ethionine/ml. Five μmoles L-methionine were injected along with growth factor at the times indicated. ———, inositol-requiring; – — – —, vitamin B_6-requiring; – – – – –, leucine-requiring.

Fig. 8. Effects of storage at −10° on the viability of *Neurospora crassa* conidia incubated for 2 hr. in minimal medium before freezing. Viability was determined by colony count after thawing and plating the suspensions. Curve (1): inositol-requiring; curve (2): wild-type; curve (3): leucine-requiring; curve (4): inositol-requiring incubated in buffer.

During the course of these experiments it was noted that the behaviour of conidia after freezing at the temperature of acetone-dry ice mixture and then storing at −10° depends on the metabolic condition of the conidia. Wild-type conidia incubated for 2 hr. in minimal medium before freezing gradually died on storage at −10°, in contrast to non-incubated controls. The inositol-requiring mutant mimicked the behaviour of the wild-type (Fig. 8), but conidia of the leucine-requiring mutant remained viable after freezing and storage at −10° for the length of the experimental period. Incubation must be in minimal medium since conidia of the inositol-requiring mutant incubated in buffer show no difference in survival after freezing from their non-incubated controls.

DISCUSSION

If the strains studied are typical then the 'suicide' effect must be related to the start of growth in those strains which kill themselves. The inositol-requiring strain, used as an example of a mutant which commits suicide in minimal medium, starts to synthesize protein and nucleic acid, its nuclei divide, and a certain proportion of the spores send out germ tubes. In contrast, the leucine-requiring strain, which does not commit suicide, carries out none of these activities during incubation for 6 hr. There is no difference between the ability of conidia of these two strains to oxidize carbohydrate (Table 4) and therefore the differences between them are more likely related to their different synthetic capacities. The effect of ethionine and of fluorophenylalanine in prolonging survival is most easily explained in terms of this hypothesis. We suppose that ethionine prolongs survival by temporarily preventing the initiation of synthetic processes, thereby making conidia of the inositol-requiring and other 'suicidal' mutants similar to those which do not kill themselves off. In essence, the mutants incubated in the presence of growth factor analogue behaved in the same way as the double mutants prepared by Macdonald & Pontecorvo (1953). With some exceptions these double mutants show increased survival on incubation in minimal medium as compared to the single mutant which dies off most rapidly.

We believe that the data contain clues to the questions (*a*) why are only actively-synthesizing spores susceptible to death by starvation, and (*b*) what is the difference between strains which do or do not commit suicide? Assuming that the genetic blocks in these mutants are complete, or almost so, any growth which occurs in minimal medium must be due to material that has been stored in the conidium. A rough idea of the amount of growth that is likely to be obtained might then be obtained from the growth-response curves. Strains that have only a small growth requirement are more likely to have stored sufficient growth substance in their conidia to initiate growth. The strain with the smallest growth requirement (vitamin B_6-requiring) germinates most readily in minimal medium, whereas the strain with the highest growth requirement (leucine-requiring) does not germinate at all during the time period of these experiments (Figs. 2, 4). There is a similar correlation between growth requirement and the suicide effect. The strain with a low growth requirement also kills itself rapidly; the adenine-requiring strain, with an intermediate-high growth requirement, dies only slowly and the strains with the highest quantitative growth requirement (i.e. the amino acid-requiring strains) do not die at an appreciable rate.

This analysis of the relation between growth requirement and the suicide effect is confirmed by the experience of workers using the filtration-selection technique (Woodward, De Zeeuw & Srb, 1954) for mutant isolation (Drs Srb and Giles, personal communications). This technique is excellent for the isolation of amino acid-requiring mutants, but the yield of vitamin-requiring mutants is low and Dr Giles has not obtained inositol-requiring mutants by this procedure. Since the method requires incubation of mutant spores in minimal medium for long periods, the difficulty in isolating mutants with low growth requirements is easily understood.

The hypothesis requires the supposition that non-germinated conidia differ in some significant way in their response to starvation from conidia which have started to synthesize. While we have no idea as to why this must be so the data show that there is a major difference in the response of the organism to its environment in these two different physiological states. Once synthesis has started, conidia are susceptible to death when kept at low temperature; when synthesis has not started, conidia of the same strains are resistant to cold death (Fig. 8). It is not the freezing *per se* which causes death but rather the storage period, since the strains used show no loss of viability if they are thawed and plated on growth medium immediately after freezing. The cold death phenomenon cannot be explained as due to mechanical effects caused by the absorption of water during the incubation period with the subsequent formation of ice crystals since (*a*) incubation of the inositol-requiring strain in buffer did not lead to a decreased resistance to cold storage, and (*b*) incubation of the leucine-requiring mutant in minimal medium did not lead to a decreased resistance even though, as has been shown above (Table 4), the leucine-requiring mutant oxidizes sugar readily in minimal medium. The cold death phenomenon must therefore be related to synthetic metabolism. Interruption of synthetic processes (by cold) once begun is lethal for the wild-type and for the inositol-requiring mutant.

There is one major exception to the correlation between growth initiation and the suicide effect. The succinate-requiring mutant, *at suc*, dies on minimal medium after incubation for 2 days. It is not possible to detect any significant increase in nucleic acid or protein and there is no germination of this strain, after incubation in minimal medium. We do not feel that this exception refutes the hypothesis. It has previously been reported (Strauss, 1956) that there is an unstable balance between carbohydrate and nitrogen metabolism in the succinate-requiring mutants; ammonium salts inhibit the *at suc* mutant and lead to the accumulation of keto acids and acetylmethylcarbinol. On minimal medium therefore, the *at suc* strain is unbalanced in its degradative metabolism in contrast to the other strains. The accumulation of products and the inhibition of the carbohydrate oxidation of this strain in the presence of ammonium salts might reasonably be expected to lead to cell death even in the absence of protein and nucleic acid synthesis.

We therefore feel that the death of mutants of Neurospora when incubated on minimal medium is a case of ‘unbalanced growth’. Starvation for a component in Neurospora, is only effective as a cause of death when active synthesis is proceeding. This must mean that death ensues when the reaction pathways which utilize the missing or limiting component cannot ‘keep up’ with the other reaction pathways and this in turn may reasonably be defined as a condition of imbalance. Active synthesis in the absence of the required growth factor is necessary but apparently not sufficient to lead to cell death. A thiamine-requiring mutant of Neurospora germinated in minimal medium but did not die after 7 days’ incubation on minimal medium, and a pantothenic acid-requiring mutant germinated but did not start to die off rapidly until after 5 days’ incubation on minimal medium. There appears to be something specific about the factors which lead to imbalance and death. This fact was clearly recognized by Macdonald & Pontecorvo (1953) as a result of their survey of mutant types.

Assuming that the cause of death in Ophiostoma and in Aspergillus mutants is similar to that in Neurospora, cell death due to 'unbalanced growth' has been recorded in three fungi as well as in *Escherichia coli* (Cohen & Barner, 1954). In the case of the fungi at any rate this death is not restricted to cells requiring DNA constituents. Our use of the term 'imbalance' is only intuitive and is as imprecise as the use made of it by Cohen & Barner (1954). It is quite evident in their case, as in ours, that there is an imbalance but it is far from clear why such an imbalance should lead to cell death.

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The Effect of Inoculum Size on Inhibition Zones in Agar Media Using Staphylococci and Streptomycin

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SUMMARY: Investigations on the size of inhibition zones in agar tube cultures of *Staphylococcus aureus* (Mayo) due to diffusion of streptomycin have confirmed the formula previously suggested. No significant deviation from expectations has been found over a temperature range of 27–40°, nor with variations of inoculum size from 2×10^3 to 5×10^8 organisms/ml. The theoretical basis of the formula has been further elucidated and the meaning of *critical population*, *critical concentration* and *critical time* clarified. These constants are determined by the necessity of absorbing a *critical amount* of antibiotic on the organisms to inhibit growth within the zone. To maintain this absorption a minimum inhibitory concentration must remain in the surrounding medium.

Cooper & Woodman (1946) proposed, on theoretical grounds, a formula for the size of antibiotic inhibition zones in agar media and Cooper (1955) extended this formula by elucidating the nature of the factors which control the time during the growth of the organism at which the size of the zone is determined. This *critical time* T_0 is given by:

$$T_0 = L + G \log_2 (N'/N_0) = X^2/4D \log_e (m_0/m'),$$

where L = lag period in viable count; G = generation time; N_0 = number of organisms in inoculum/unit volume final medium; N' = *critical population* reached at time T_0 after inoculation, per unit volume of agar; X = depth of inhibition zones; D = diffusion coefficient of antibiotic; m_0 = concentration of antibiotic in contact with the agar surface in the tube; m' = *critical concentration* at distance X from surface at time T_0.

The conditions under which this theoretical formula is expected to be true are necessarily those under which the initial assumptions are most nearly approached. The most important are: an antibiotic which diffuses freely through a non-interfering medium from a reservoir of constant concentration, and an inoculation of a determined number of metabolizing organisms which, after a constant lag period, multiply at a constant rate. Constancy of temperature and of media composition are also important. Many workers have examined the validity of the formula of Cooper & Woodman (1946) which connects the zone size with the diffusion laws and critical time; these include Vesterdal (1947), Masuyama (1948), Herdan (1949), Linton (1949), Sykes, Lewis & Goshawk (1951), Miyamura (1953), Schonfeld (1953), Ingram, Foxell & Armitage (1953), Schlesinger (1954) and Gillissen (1954). Others have, in addition, contributed to our knowledge of the relationship between critical time and the growth of the organism. These are Mitchison & Spicer (1949), Cooper & Gillespie (1952), Cooper & Linton (1952), Cooper (1952), Humphrey

& Lightbown (1952), Linton (1954, 1955), Cooper (1955). In the first paper of Cooper & Woodman (1946) it was shown that zones could be abolished by pre-incubating cultures before beginning the diffusion of the antibiotic. When growth proceeds for h hr. after time 0, before the antibiotic is added, the time of diffusion is given by

$$T_0 - h = X^2/4D \log_e (m_0/m').$$

When $h=0$ (the usual condition in assays), then the time of diffusion becomes the same as the time of growth, T_0. When $h=T_0$, then growth has reached the critical population N' before diffusion starts and no zone of inhibition is produced. When the size of the inoculum (N_0) is increased, then the time T_0 required to reach the critical population should be diminished. A critical survey of the influence of inoculum size upon the size of inhibition zones at different temperatures has hitherto not been published; this is the object of the present paper. We have investigated this for the action of streptomycin on sensitive and resistant strains of *Staphylococcus aureus* in a constant medium.

METHODS

Organisms. (i) The 'Mayo' strain of *Staphylococcus aureus* was used as a streptomycin-sensitive organism (our collection reference No. 6473). This strain was kept freeze-dried and retained a constant m' value of 2·5 μg. streptomycin/ml. Earlier experiments with this strain before freeze-dried stocks were kept showed slightly greater sensitivities with an m' value as low as 1·8 μg./ml. (see Table 1).

(ii) A resistant mutant, with the same growth rate as the 'Mayo' staphylococcus, was derived from this strain. This also was freeze-dried and retained a constant m' value of 7·08 μg. streptomycin/ml. (our collection reference No. 6474).

For a series of experiments both strains were maintained on nutrient agar slopes, subcultured at weekly intervals and kept at room temperature. A fresh culture from a freeze-dried ampoule was used when the m' value was found to alter as a result of repeated subculture.

Standard broth was used in the preparation of the inocula and for the determination of generation time and lag periods. It was prepared with the following ingredients: Bacto-peptone, 10 g.; NaCl, 5 g.; beef heart infusion made with glass-distilled water, 500 ml.; and the volume made up to 1000 ml. with glass-distilled water. After adjusting to pH 7·8, it was dispensed in screw-cap bottles (100 ml. quantities) sterilized by autoclaving and stored in the dark.

Standard agar. For diffusion experiments, the standard broth was gelled with 1% (w/v) Bacto-agar.

Antibiotic. Streptomycin calcium chloride (Glaxo) was diluted in sterile glass-distilled water to give a final concentration of 200 mg. streptomycin/ml. This was stored at 4° in screw-cap bottles and replaced at fortnightly intervals. Dilutions were made from this stock solution in sterile glass-distilled water immediately before each experiment was set up, to contain 10, 100 and 1000 μg. streptomycin/ml.

Preparation of inocula for diffusion experiments. The organism to be tested was inoculated into 10 ml. standard broth in a plugged tube, and incubated for 20 hr. at 37°. Immediately before beginning the experiment, this culture was diluted in sterile glass-distilled water warmed to 37°, to give a series of dilutions. These were planned so that when 0·5 ml. was added to 9·5 ml. of molten standard agar, held at 45° in a water bath, the final number of viable organisms approximated to the required counts. The viable count of the 20 hr. broth culture was determined by the technique of Miles & Misra (1938) for each series of experiments and the size of each inoculum was calculated from this.

Diffusion experiments

Procedure. The assay method of Mitchison & Spicer (1949) was used, with certain modifications described by Cooper & Gillespie (1952). By this method, molten standard agar, after inoculation, was immediately pipetted into sterile glass tubes (3×75 mm.), to a depth of *c.* 40 mm. Mitchison & Spicer showed that variation of degree of anaerobiosis by alteration of depth of column was without influence on the size of the zones in the case of streptomycin. The tubes were placed in racks in the upright position and the agar allowed to set by standing at room temperature for 10 min. The racks of tubes were then incubated in water baths regulated at the required temperatures. The time at which incubation was begun was regarded as time 0. Three concentrations of streptomycin (10, 100, 1000 μg./ml.) were pipetted on to the agar surface of the first of three series of tubes at time 0 and, thereafter, at convenient periods of pre-incubation, e.g. 1, 2, 3, etc. hr. The depths of the zones of inhibition, produced by diffusion of the streptomycin, were read after 20 hr. incubation. The square of the values (X^2) thus obtained, for each concentration of streptomycin and for each inoculum size, were plotted in various ways in order to determine the constants T_0, m' and D in the formula (Cooper, 1955).

Determination of T_0. When the values of X^2 were plotted against the time of pre-incubation (h) it was found that, independent of the concentration of streptomycin, straight lines which connected the experimental points intersected the h axis at time T_0 hr. This value was determined for each inoculum size (N_0) investigated, over a range from 2×10^3 to 5×10^8 viable organisms/ml.

Determination of m'. Plotting the values of X^2 against the logarithm of the concentrations of streptomycin used in the experiments (10, 100, 1000 μg./ml.) resulted in straight-line graphs being obtained for each value of h and these intersected on the concentration axis at m'.

Determinations of D. This constant was calculated by substituting experimental values of X^2, T_0, m^0 and m', obtained at 35°, in the formula

$$T_0 = X^2/4D \log_e (m^0/m').$$

Inocula of different sizes were used over the whole range possible and the mean value of a large number of determinations was adopted. Values of D for other temperatures were obtained theoretically, by correcting the value at 35° for the change in the viscosity of water with temperature, by means of the formula:

$$D_1/D_2 = T_1\eta_2/T_2\eta_1,$$

where D_1 and D_2 are the diffusion coefficients at absolute temperatures T_1 and T_2 and η_1 and η_2 are the viscosities of water at these temperatures (see Cooper & Woodman, 1946).

The effect of inoculum size on inhibition zones. In order clearly to demonstrate this, the experimental values of X^2 for $h=0$ have been plotted against the logarithm of the inoculum size, $\log_2 N_0$ (Figs. 1*b*, 2*b* and 3*b*). This was done with the sensitive staphylococcus for each concentration of streptomycin at one temperature (Fig. 1*b*) and similarly with the resistant mutant (Fig. 3*b*). Results are given for one concentration of streptomycin at different temperatures (Fig. 2*b*). In addition the T_0 values determined under the same conditions have been plotted against the logarithm of the inoculum size (Figs. 1*a*, 2*a* and 3*a*).

Determination of generation time and lag periods

Broth experiments. The generation time and lag periods were determined in standard broth for both the sensitive and resistant staphylococci and at each temperature at which the diffusion experiments were done. These constants were determined by using inocula of approximately $1{\cdot}0 \times 10^6$ viable organisms/ml., and under conditions as similar as possible to those of the diffusion experiments. This included diluting a 20 hr. broth culture in sterile glass-distilled water (1/50), adding 0·5 ml. of this to 9·5 ml. of standard broth warmed to 45°, cooling at room temperature for 10 min. and then beginning incubation in regulated water baths. The generation time and lag periods were determined by viable counts on samples removed at regular intervals.

Agar experiments. An attempt was made to determine the generation time in solid medium. Inoculated tubes of standard agar were prepared as for diffusion experiments and incubated without streptomycin. At intervals of time, tubes were taken from the water bath and their sealed ends cut off with a glass file to facilitate removal of the agar columns. These were fixed in formalin for 12 hr. and washed in distilled water for a further 4 hr. They were then floated on to glass slides, dried at 37° in the incubator and stained by Gram's method. The number of organisms which had developed in each colony were counted by direct microscopy. Though the method was not very accurate, reasonable agreement was obtained with the determinations in broth.

RESULTS

Many experiments were undertaken with a view to determining quantitatively the effect of heavy and light inocula on the size of inhibition zones formed by diffusion of inhibitory concentrations of streptomycin. Only some examples of the results can be given here and are illustrated by Figs. 1–3.

Figures 1*a* and 1*b* illustrate the results obtained at 35°, with the sensitive 'Mayo' staphylococcus. For each inoculum size used, *m′* was determined, and the value of 1·8 μg. streptomicin/ml. was found to be constant throughout the series. The extrapolated values of T_0 were separately determined for each inoculum size and became smaller as the inoculum size was increased. Each value of T_0 was obtained from experimental readings of X^2 for all periods of preincubation (h), from nine to twenty-seven readings being used to determine each point. The values of T_0 have been plotted against the logarithm of the inoculum size, $\log_2 N_0$ (Fig. 1*a*) and the best straight line through these points intersects the inoculum size axis at $\log_2 N_0 = 27{\cdot}0$ ($N = 135{\cdot}5 \times 10^6$ organisms/ml.). Inocula of greater size than this are too great to allow the formation of inhibition zones, and, therefore, $T_0 = 0$ in these cases.

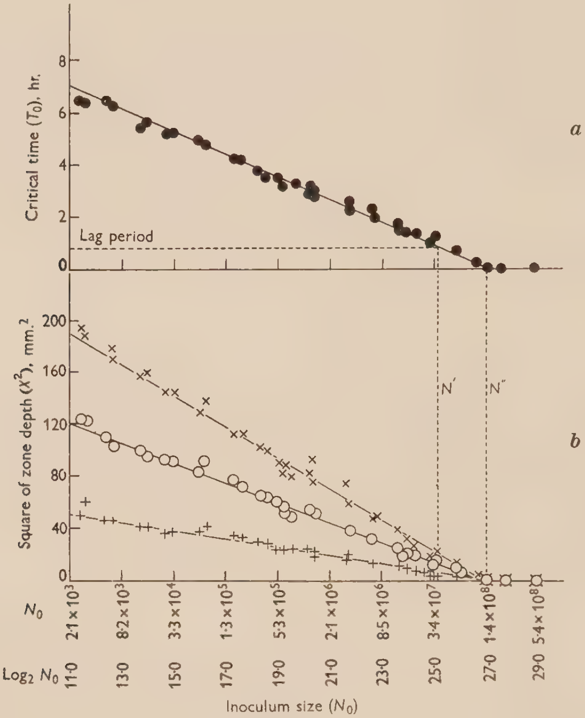

Fig. 1. The effect of inoculum size on critical time and the depth of inhibition zones produced by the action of streptomycin on staphylococcus (Mayo 6473) at 35°. (*a*) Critical time (T_0) plotted against inoculum size ($\log_2 N_0$). The points are the extrapolated values of h obtained from graphs plotting zone size (X^2) against hours of preincubation (h), since when $X^2 = 0$, then $h = T_0$. Three lines for different concentrations of antibiotic ($m_0 = 10$, 100, 1000 μg./ml.) were extrapolated and the average value used for each size of inoculum tested (●). (*b*) Zone size (X^2) determined in triplicate experiments, for m_0 values of 10 μg./ml. (+), 100 μg./ml. (○), 1000 μg./ml. (×), when $h = 0$ plotted against inoculum size. N' is the critical population, which all inocula less than N' achieve at time T_0 when the zone edge is formed. N'' is that size of inoculum ($N_0 = N''$) which reduces T_0 and X^2 to nil. For theoretical constants used to calculate the graphs, see Table 1.

In Fig. 1*b*, the values of X^2, obtained with each of three concentrations of streptomycin (10, 100, 1000 μg./ml.), are plotted against the logarithm of the inoculum size. It is seen that the size of the inhibition zones is decreased to zero when the inoculum size is given by $\log_2 N_0 = 27{\cdot}0$. This value of N_0, which is independent of the concentration of the antibiotic used, we have represented by N''.

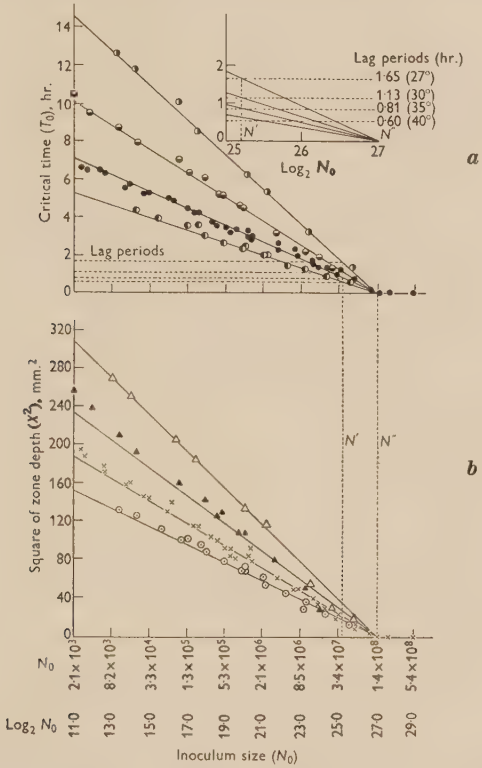

Fig. 2. The effect of inoculum size on critical time and the depth of inhibition zones produced by the action of streptomycin on staphylococcus (Mayo 6473) at different temperatures. (*a*) As in graph 1*a*. When $N_0 = N'$ then $T_0 = L$, and the inset graph shows the theoretical values of L at these temperatures. 27°, ◑; 30°, ◒; 35°, ●; 40°, ◐. (*b*) As in graph 1*b*, but values of X^2 are plotted for one value of m_0 ($=1000$ μg./ml.) only, for each temperature. 27°, △; 30°, ▲; 35°, ×; 40°, ⊙.

The calculation of regression lines through the points for $\log_2 N_0$ plotted against T_0, gives a value of N'' which $\pm 2\sigma$ (σ = standard deviation) overlaps N'' values calculated from the regression lines of the X^2 values for each concentration. N'' is thus independent of the concentration of the antibiotic and within the limits of experimental error lies on the extrapolation of the best straight lines determined for any workable range of sizes of inocula. The value $\log_2 N'' = 27{\cdot}0$ has been chosen as the most probable value for all our experiments, including those at temperatures other than 35°.

The value of T_0 was analysed by Cooper & Gillespie (1952) and Cooper & Linton (1952), and includes the time taken for the inoculum to reach a critical population by cell division, together with the lag period of growth. Since the generation time and lag period were determined for the 'Mayo' staphylococcus by broth experiments (see Table 1), it was possible to show this value on Figs. 1*a* and 1*b*. By erecting a vertical line, from the point of intersection with the lag period on the time axis in Fig. 1*a* to the inoculum size axis, the critical population is shown as $\log_2 N_0 = 25{\cdot}2$ ($N_0 = 39{\cdot}9 \times 10^6$ organisms/ml.). This value of N_0 we have called N'.

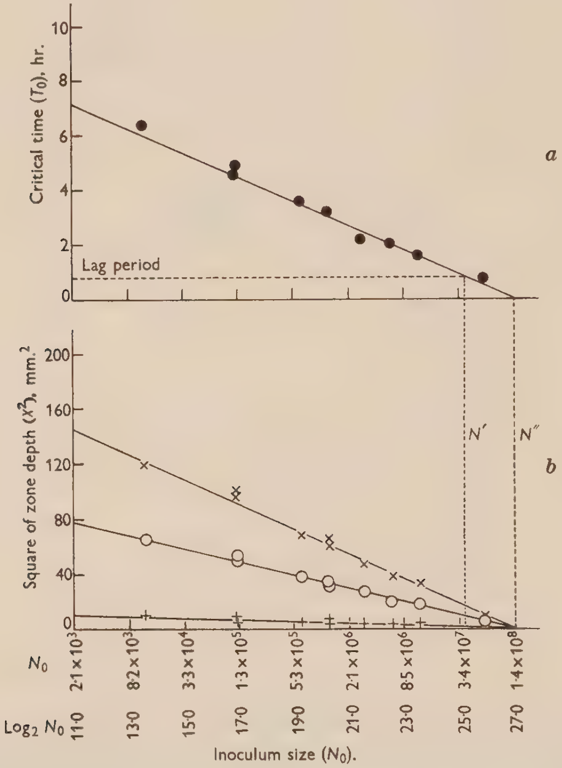

Fig. 3. The effect of inoculum size on critical time and the depth of inhibition zones produced by the action of streptomycin on staphylococcus, resistant strain, 6474, at 35°. As in graph 1 (*a* and *b*). This strain has approximately the same growth rate, over the temperature range 27–40° (see Tables 1 and 2), but a higher resistance to streptomycin ($m' = 7{\cdot}08$ μg./ml.) which was unaffected by temperature.

The straight-line graphs in Fig. 1*b* have been calculated by substituting the experimental values of T_0, m', m_0, D, N_0 and N' in the formulae proposed by Cooper (1955). These are

$$X^2 = 4D\,(\log_e m_0/m')\,T_0, \tag{1}$$

and

$$X^2 = 4D\,(\log_e m_0/m')\,(L + G\log_2 N'/N_0). \tag{2}$$

From formula (1) it is seen that

$$T_0 = X^2/4D\log_e (m_0/m'), \tag{3}$$

and this is derived from the laws of diffusion and the conception of critical concentration at this time. From formulae (1) and (2) it is seen that

$$T_0 = L + G\log_2 (N'/N_0), \tag{4}$$

and this is derived from the laws of growth of the organism, and gives the time required to achieve a critical population, N'.

The agreement between the experimental values of X^2 and the calculated graphs is excellent.

The influence of temperature

In addition to the influence of inoculum size on inhibition zones, the effect of temperature was next investigated. Experiments were conducted at 27°, 30°, and 40° for comparison with those at 35°, with the same test organism. Three concentrations of streptomycin were used (10, 100, 1000 μg./ml.). Fig. 2*a* shows the experimentally determined values of T_0 for each temperature, plotted against the logarithm of the inoculum size. The best straight lines through these points intersect the inoculum size axis at $\log_2 N_0 = 27{\cdot}0$. In Fig. 2*b* the experimental value of X^2 for each temperature with one concentration of streptomycin (1000 μg./ml.) is plotted against the inoculum size. Fig. 2*a* and *b* show that whilst the slopes of each series of graphs vary with temperature, the N'' value remains constant. By comparing the lag periods experimentally determined in broth cultures, with those calculated and used in Figs. 2*a* and 2*b*, it is shown that very good agreement was obtained (Table 1). The critical population (N') is thus also constant and independent of temperature. This is shown more clearly by the enlarged inset graph.

Table 1. *Streptomycin and a sensitive staphylococcus*

The value of each variable determined experimentally and used for the theoretical calculations of graphs $X^2/\log_2 N_0$ with streptomycin and sensitive strain of *Staphylococcus aureus* (Mayo 6473).

			Critical concentration†	Zone-size constants in agar		Growth-curve constants in broth	
Temp.	Viscosity* 10^2 c.g.s.	Diffusion coefficient D mm.²/hr.	m' μg./ml. (average)	L (hr.)‡	G (hr.)	L (hr.)	G (hr.)
27	0·855	0·878	2·5	1·65	0·91	1·65	1·35
30	0·800	0·948	2·1	1·13	0·625	1·125	0·75
35	0·728	1·06	1·8	0·81	0·45	0·825	0·575
40	0·657	1·19	2·5	0·60	0·33	0·60	0·42

L/G Av. ratio 1·81‡

Theoretical value used for the graphs:

$\log_2 N' = 25{\cdot}2$ $\qquad$ $\log_2 N'' = 27{\cdot}0$

$N' = 38{\cdot}9 \times 10^6$ $\qquad$ $N'' = 135{\cdot}5 \times 10^6$

* Values quoted by Kaye & Laby (1944).

† The deviations in m' with temperature were not experimentally significant. The variations recorded here are due to varied sensitivity on repeated subculture—but analogous variations occurred at 35° as at other temperatures with the same strain. The average values in the table were used for theoretical graphs.

‡ The average ratio of L (in broth) to G (in agar) over the whole temperature range was used to calculate these L values in agar.

The values of the constants used to calculate the graphs in Fig. 2*b* are given in Table 1. The results reported in this paper have been obtained over a period of about two years, and it was found necessary to check repeatedly the sensitivity of the test strain to streptomycin. This was done by control determination of m' at 35°. When the m' value was found to have altered after repeated subculture, a freeze-dried ampoule of the strain was opened for further tests. The slight variations in the m' value of the test strain in Table 1 were due to an increased resistance found after certain subcultures before the freeze-drying technique was introduced.

Table 2. *Streptomycin and a resistant staphylococcus*

The values of each variable determined experimentally and used for the theoretical calculations of graphs $X^2/\log_2 N_0$ with streptomycin and resistant strain of *Staphylococcus aureus* (Mayo 6474).

Temp. (°C.)	Viscosity* 10^2 C.G.S.	Diffusion coefficient D mm.²/hr.	Critical concentration† m' μg./ml. (average)	Zone-size constants in agar: L (hr.)‡	Zone-size constants in agar: G (hr.)	Growth-curve constants in broth: L (hr.)	Growth-curve constants in broth: G (hr.)
27	0·855	0·878	7·08	1·65	0·91	1·65	1·35
30	0·800	0·948	7·08	1·13	0·625	1·125	0·77
35	0·728	1·06	7·08	0·825	0·45	0·825	0·575
40	0·657	1·19	7·08	0·60	0·33	0·60	0·45

L/G Av. ratio 1·81‡

Theoretical values used for the graphs:

$\log_2 N' = 25{\cdot}2$ $\quad$ $\log_2 N'' = 27{\cdot}0$

$N' = 38{\cdot}9 \times 10^6$ $\quad$ $N'' = 135{\cdot}5 \times 10^6$

* Values quoted by Kaye & Laby (1944).

† The strain remained quite consistent in its m' values throughout these series of experiments. Whenever a change was noticed, a new ampoule from the freeze-dried stock was obtained.

‡ The average ratio of L (in broth) to G (in agar) over the whole temperature range was used to calculate these L values in agar.

Experiments with the resistant mutant

The effect of inoculum size at different temperatures was determined with the resistant staphylococcus mutant. This strain had the same growth rate as the sensitive parent strain but an increased m' value equal to 7·08 μg. streptomycin/ml. The experimental results were plotted in the same manner as described for the sensitive strain. Figure 3 shows the results at 35°, and it will be seen that, whilst the X^2 values are markedly reduced, the N' and N'' values are the same as for the sensitive staphylococcus. The analogous results obtained at other temperatures are summarized in Table 2. The use of a freeze-dried standard enabled the value of m' to be kept constant for all experiments.

Generation time

The slope of the graphs in Figs. 1*a*, 2*a* and 3*a* are dependent upon the generation time (G) of the test organism and therefore given a direct reading of this constant. These slopes were calculated from regression lines for the experimental points between $\log_2 N_0 = 15{\cdot}0$ and $22{\cdot}0$, because zone edges in this range can be most accurately determined (see Table 1). A comparison between these values in solid media compared with determinations in broth shows the value for G in agar was only 80% approximately of that obtained by broth experiments. Possible reasons for this are suggested later.

DISCUSSION

The significance of critical time T_0

Cooper & Woodman (1946) and Cooper & Gillespie (1952) showed that pre-incubation of cultures decreased zone size. When the time interval between beginning incubation and adding the antibiotic (h) became equal to T_0 the zone size diminished to zero, no matter what the concentration of antibiotic used (m_0). With a standard size of inoculum, T_0 varied according to the temperature of incubation in such a way that the same number of generations resulted, a constant population (N') being obtained. It is now shown that no matter what size of inoculum (N_0) is used at time 0, the length of T_0 is varied, so that this same critical population (N') is achieved. At time T_0 the critical concentration m' fails for the first time to inhibit further growth.

When the graphs plotting T_0 against $\log_2 N_0$ (Figs. 1*a*, 2*a* and 3*a*) are examined it will be seen that T_0 diminishes with increasing inoculum size. The experimental points agree well with formula (4):

$$T_0=L+G\log_2(N'/N_0). \tag{4}$$

When $N_0=N'$, then $T_0=L$, since $\log N'/N_0=0$. With values of $N_0>N'$, the value of T_0 becomes less than L, and the experimental points agree with the prolongation of the straight line until it meets the axis at N'' when $T_0=0$. This holds true for all values of h, including $h=0$.

Equation 4 may be written

$$T_0/G=L/G+\log_2(N'/N_0) \tag{4a}$$

and when $N_0=N''$ then $T_0=0$ and $0=L/G+\log N'/N''$

$$L/G=\log N''-\log N'. \tag{5}$$

Eliminating N' from equations (4*a*) and (5)

$$T_0=G\log N''/N_0. \tag{6}$$

With an inoculum size of N'', the cocci are able to remove streptomycin to such an extent that the inhibitory concentration is not reached and growth occurs, giving no inhibition zones. With an inoculum of N' cocci we get inhibition zones. Their size shows they are formed within the lag period (L). Cocci to the number of N', at the end of the lag period, combine with, and are inhibited by, the same amount of antibiotic as the larger number of cocci N'' at time 0. The cocci showed no difference in sensitivity (m') whatever the size of the inoculum, including populations with N_0 falling between N' and N''. It follows, therefore, that from time 0 to time L, the combining power of the cocci for streptomycin is increased, and as the experimental data agree with a straight-line graph up to $N_0=N''$ it seems certain that the full metabolic rate governing the synthesis of streoptomycin-absorbing sites has been achieved at time 0 in our experiments. It is known that under ideal conditions metabolic lag may be abolished, even though cell division lag remains (Gale, 1943).

We find experimentally, for the staphylococcus used, that both N' and N'' prove to be constant even at different temperatures (Fig. 2). Since from equation (5) $\log N'' - \log N' = L/G$ this could only be so if L/G was constant for all temperatures. Our experimental determinations of L and G in broth did give an approximate constant ratio up to 40° C. The relative values of L agreed with the T_0 values for $N_0 = N'$ at all temperatures. (See Tables 1 and 2.)

Table 2 shows that analogous results were obtained with a resistant mutant, chosen because it had the same growth rate as its prototype. The values of all constants in the formula remain the same as in Table 1, except the value of m'. The value of T_0 is entirely determined by the time required for the population to reach N' per ml. and, as the theory predicts, is quite independent of m'.

The generation time G

Johnson & Lewin (1946) showed in the case of *Escherichia coli* that the variation of generation time with temperature was made up of two parts:

(*a*) Over the lower temperature range, growth occurred as though limited by the synthesis of one enzyme system, and the rate increased with temperature as would be expected from the thermodynamic constants involved.

(*b*) At higher temperatures, enzyme denaturation competed with enzyme synthesis and eventually lessened growth with increasing temperature. Heat denaturation when prolonged or excessive became irreversible; it may, however, be reversible but be more difficult to detect over short periods.

Apparently enzyme denaturation in our experiments is of little importance during the period of lag and the first few generations. Enzyme synthesis is thus the important consideration governing the formation of streptomycin-absorbing sites concerned in the formation of inhibition zones. Johnson & Lewin (1946) showed that by plotting rate of growth (as percentage of maximum rate) against the reciprocal of the absolute temperature, a falling straight line resulted at low temperatures, a rising one at high temperatures, whilst middle temperature ranges were the resultant of the two.

When our values of G determined from zone size were plotted in this way, the experimental values fell on a straight line. This would be expected if streptomycin combined with protein before denaturation or if no protein denaturation occurred. Evidently the full rate of protein synthesis was established by time 0, and the lag period in cell division was proportional to this rate, so that L/G was constant. Values of G may be calculated from antibiotic zones with considerable accuracy especially if T_0 is determined for light inocula. However, the value of G calculated from the slope of the zone-size graphs was only 80% of the generation time determined from viable counts on broth cultures. A number of alternative explanations are possible; the true reason of this difference has not yet been determined. Most probably the greater size of the cocci during the early hours of growth is the cause (see Hershey, 1938, 1939). The absorption of toxic substances by agar might however contribute.

The significance of critical populations N′ and N″

The absorption of antibiotics by bacteria has been investigated by many workers. Berkman, Henry, Housewright, & Henry (1948) showed that absorption of streptomycin by staphylococci reaches equilibrium rapidly, and that streptomycin could be completely eluted with NaCl solution. The amount of streptomycin absorbed and recovered was identical with resistant and susceptible strains, though the concentrations necessary in the external medium to inhibit growth, might be as different as 1 and 1000 μg. streptomycin/ml. Eagle, Levy & Fleischman (1955) showed by using radioactive penicillin that when inhibitory or lethal effects were produced, different species of bacteria of various degrees of susceptibility quantitatively accumulated the same amount of penicillin per organism. Sensitive strains were able to absorb from low concentrations in the medium, resistant strains only absorbed the same amount from higher concentrations. The plots of their results resemble Langmuir absorption curves, theoretically obtained by saturating more and more receptors in or on the organism until all were saturated, inhibition of growth (multiplication) occurring before this point. Some of their strains of staphylococcus (e.g. strain VI) showed a capacity for much further absorption (with higher concentrations) above this point. Humphrey & Lightbown (1952) found that the absorption of streptomycin by staphylococci follows the Freundlich type ($X = 20{\cdot}4\,C^{0{\cdot}24}$) over the range of concentration examined, though saturation is expected to be achieved at higher concentrations (see Clarke, 1933). The electrophoretic mobility of staphylococci with absorbed streptomycin (studied by McQuillen, 1951), showed agreement with the Langmuir isotherm. A certain minimum external concentration is necessary to saturate sufficient active sites in order to inhibit division and in the case of staphylococci and streptomycin is reached before saturation. The critical concentration m' in diffusion experiments, necessary to prevent further division, is some 2–3 times greater than the minimum inhibitory concentration which will prevent growth when incorporated in broth or agar using a small inoculum. This is because part of the antibiotic (that which causes inhibition) is removed by absorption on the organisms which, under diffusion conditions, are initially present in excess at the zone edge. The external concentration is thus lowered to the minimum inhibitory concentration. The value of m' thus represents the sum of the absorbed and free streptomycin. When incorporated in the medium from the start, antibiotic is in excess and the external concentration is less affected when antibiotic is removed by the organisms of a small inoculum. The critical concentration m' is only capable of saturating enough active sites to inhibit growth in a limited number of organisms, namely N' organisms during logarithmic growth and N'' organisms at the moment of inoculation, i.e. organisms harvested from a culture which develops a lag period before regular cell division.

In agreement with the above considerations the values of N' and N'' have remained constant, even when the value of m' has been varied considerably by methods expected to require greater external concentrations to achieve the same concentration within the organisms. This has been done (1) by using a mutant variant of our staphylococcal strain, which had much less sensitivity to streptomycin but the same growth rate at each temperature (see Fig. 3 for results at 35°), (2) by incorporating $MgSO_4$ in the medium. This salt is a competitive antagonist for streptomycin absorption and experimental values of m' for variations of Mg from 66 to 5000 p.p.m. allowed m' to be varied from 1·5 to 25·0 μg. streptomycin/ml. in our medium (Linton, 1954). Results are expressed in terms of Mg concentration, but the sulphate ion is also important in this competition. The value of m' has been increased by this method up to 16 times without affecting T_0, so presumably the values of N' and N'' were unchanged.

The significance of N' and N'' in diffusion experiments is therefore that these are the number of cocci/ml. (young and old respectively) which by absorption of streptomycin can be inhibited by m' μg. streptomycin/ml. because after absorption the external concentration falls to the minimum inhibitory concentration as measured by incorporation experiments.

The significance of critical concentration m'

We have for reasons of simplicity assumed that the streptomycin arriving by diffusion at an organism acts instantaneously, and if the concentration $< m'$ is without effect, if the concentration $> m'$ inhibits further cell division; such an assumption is obviously a mathematical approximation. Absorption of antibiotic by cells takes time, though the time to reach equilibrium between absorbed streptomycin and a particular concentration in the external medium is short—less than 10 min. according to Humphrey & Lightbown (1952). This period is less than the shortest possible generation time, and we may therefore consider that the absorbed antibiotic will inhibit the next cell division when the external concentration is still high enough to be at the minimum inhibitory concentration as determined by incorporation experiments in broth or agar.

Let a = amount of antibiotic removed from the medium by one coccus.

Then $N''a$ = amount of antibiotic removed from 1 ml. of medium by N'' cocci.

Assuming that the amount of antibiotic remaining in the medium which is in equilibrium with that in the cocci is the amount required to produce bacteriostasis in the early hours of growth and that this is the minimum inhibitory concentration (C), then the critical concentration as determined by diffusion experiments is given by

$$m' = N''a + C \quad \text{or} \quad a = \frac{m' - C}{N''}.$$

Humphrey & Lightbown (1952) determined the amount of streptomycin absorbed by 1 mg. dry wt. staphylococci in different concentrations of antibiotic as given by $20{\cdot}4\,C^{0{\cdot}24}$. The number of cocci in 1 mg. dry wt. $= 3 \times 10^9$ (Burroughs Wellcome and Co., London, tables supplied with Brown's opacity tubes), so

$$a = \frac{20{\cdot}4}{3 \times 10^9}\,C^{0{\cdot}24} \quad \text{and} \quad N'' = \frac{3 \times 10^9\,(m' - C)}{20{\cdot}4\,C^{0{\cdot}24}}.$$

The average value for m' at 35° was 1·8 μg. streptomycin/ml. Estimates of minimum inhibitory concentration from growth curves, and by incorporation of streptomycin in agar media, with this strain gave $C=0{\cdot}9$ μg. streptomcyin/ml., approximately.

This gives

$$N''=\frac{3\times10^{9}\,(1{\cdot}8-0{\cdot}9)}{20{\cdot}4(0{\cdot}9)^{0{\cdot}24}}=\frac{3\times10^{9}}{20{\cdot}4}0{\cdot}9^{0{\cdot}76}=1{\cdot}36\times10^{8}$$

and $$\log_2 N''=27{\cdot}00.$$

This value is identical with our results from the effects of inoculum size.

The use of Humphrey & Lightbown's (1952) adsorption formula is justified for concentrations from 0 to 100 units, because although McQuillen (1951) by electrophoresis experiments showed that streptomycin adsorption on the surface of staphylococci fitted a Langmuir adsorption isotherm, saturation was not reached until over 1000 units were absorbed, and the part of the curve from 5 to 70 % saturation was fitted accurately by the Freundlich straight line (see Clarke, 1933).

It can thus be seen that the critical concentration of streptomycin m' diffusing within the inhibition zone, is more than adequate to supply antibiotic to the organisms and stop further multiplication if they do not exceed N'' in number. Before the time T_0 the number of cocci is insufficient to absorb enough antibiotic to lower the external concentration m' below the minimum inhibitory concentration (in this case $m'/2$), and cocci in equilibrium with such concentrations are inhibited. When more than N'' cocci are inoculated, or if by time T_0 they multiply to exceed N' in number, then the concentration of streptomycin in the external medium is lowered below the minimum inhibitory concentration. During the lag period an inoculum of N' cocci evidently develops as much absorbing capacity as N'' cocci at the moment of inoculation. No matter what the size of inoculum N_0, therefore, the critical time T_0 (when inhibition ceases) is determined by the time required for the culture to achieve a population density of N' cocci/ml. When the critical population (N' during logarithmic growth, N'' for non-dividing cocci) is exceeded, absorption lowers the value of free streptomycin below the minimum inhibitory concentration. Further growth can then occur and absorption can deal with many times the concentration m' of streptomycin because the cocci are not saturated until a concentration over 1000 μg./ml. is present in the external medium.

The lag period L

The value of L in the formula is the period from time 0 to the extrapolated point of onset of logarithmic growth. Provided active synthesis of antibiotic-absorbing sites is already fully established at time 0, the value of L may be incorporated with N' as a new population N'', containing the same number of absorbing sites at time 0 as N' population has at time L. This is only possible of course when L/G is constant. When a lag in metabolism occurs after time 0, then L must be included for, like h (pre-incubation time), it must be taken into account in determining diffusion time. It will be of opposite sign to h, because it is a time of pre-diffusion. But as Gale (1943) pointed out, increase in cell-nitrogen may show a steady increase from time of inoculation when the viable count gives a typical growth curve showing a lag phase. In this paper we have achieved such conditions and it is thus possible to eliminate L completely by using log N'' instead of $L/G + \log N'$. Nevertheless, it is important to remember, when inoculum size is determined by viable count and not by mg. dry weight, that for values $<N'$, allowance for increase in streptomycin utilization during the lag period is best made by including L in the formula. The time when cell division begins is easily determined from growth curves.

The determination of L, at different temperatures, allows N' to be accurately fixed in relation to N'' and G (see Fig. 2*a*). We have used determinations made with an inoculum size of 10^6 organisms/ml. Hinshelwood (1946) discussed under what conditions the lag period might be expected to change with inoculum size. The conditions under which we prepared our dilutions for inoculation, however, were such as would be expected to minimize any such effect. They were: (*a*) use of cocci of a constant age; (*b*) considerable dilution in all cases to abolish the effect of accumulated toxic substances; (*c*) the use of a highly nutritious medium to make the cocci independent of the need to accumulate diffusible intermediate compounds in biosynthesis.

Experimental deviations from the theory

If the explanation previously given of the difference between the value of G used in the formulae and that determined from growth curves be accepted, then no unexpected deviations from theory have been observed. We conclude, therefore, that under the conditions of our experiments our original assumptions have been sufficiently closely approached, and that the experimental observations prove the essential truth of the theory, at least for the case of the action of streptomycin on our straphylococci.

Previous work suggests that the theory is capable of accurate application to other organisms and other antibiotics, provided that the conditions of the experiments are carefully controlled to fulfil the initial assumptions made, and that no destruction of antibiotic nor lysis of organisms occurs. With suitable modifications, it may be made to apply to other conditions. For example, the formula of Humphrey & Lightbown (1952) or that of Vesterdal (1947) taking into account the falling concentration of antibiotic from a bead or small cup with a plate culture, may be used for the diffusion part of the formulae and combined with the growth curve part of our formula.

Diffusion and population

In solid media and gel-like structures such as body tissues, not only antibiotics but other toxic substances may spread by diffusion, therefore limiting growth in areas near their origin whilst being ineffective further away. The converse in which promotion of growth by diffusing nutrients occurs is, in many instances, also a means of controlling populations. The number of organisms in bacterial colonies has been shown to be a function of the distance between the colonies (Mayr-Harting, 1947), and the interchange of toxins and nutrients by diffusion necessitates relationships in time and populations. A proper understanding of the significance of critical concentration, time and population seems to us to be fundamental therefore for understanding growth and antibiotic action. The action of antibiotics in solid tissues is obviously limited by their diffusibility, but it has not, it seems to us, been realized that it is also limited by the critical population of organisms. If this be exceeded, i.e. if diffusion necessitates a critical time during which action must be achieved, then after this a thousandfold increase of antibiotic will be unable to stop further bacterial multiplication at such a site. The essential requirement of early therapy is thus shown to be a theoretical requirement as well as a clinical observation—and this is quite apart from such considerations as the development of adaptive enzymes or resistant mutants, or the destruction of antibiotic by bacterial substances.

Conclusions

(1) The formula

$$\begin{aligned} T_0-h &= L-h+G\log_2 N'/N_0 \quad \text{(growth)}\\ &= -h+G\log_2 N''/N_0 \quad \text{(metabolism)}\\ &= X^2/4D\log_e m_0/m' \quad \text{(diffusion)}\end{aligned}$$

gives the relationship between the critical time (T_0) and the size of the inhibition zone (X) in terms of the concentration of antibiotic (m), the rate of growth of the organism ($1/G$) and the number of organisms (N). Lag period of cell division $=L$, and time of pre-incubation, before adding antibiotic $=h$.

(2) The critical concentration of diffusing antibiotic (m') at the zone edge at time T_0 is partly absorbed by the cell population. The amount remaining in solution is identical with the minimum inhibitory concentration determined by inoculating a few cocci into a large volume of medium incorporating antibiotic, or by determining the bacteriostatic concentration during the early hours of growth from growth curves.

(3) When the critical concentration (m') is varied (a) by the presence of substances competing for streptomycin adsorption centres, or (b) by using resistant strains of staphylococci, the critical population is constant. This is probably because the amount absorbed/unit weight of cocci, necessary to inhibit multiplication, is constant.

(4) The critical time (T_0) is the time required for the multiplying cells to achieve the critical population (N').

(5) The critical population for non-dividing (but metabolizing) organisms (N'') is greater than for multiplying ones (N'). Even during the lag period streptomycin-absorbing sites are being synthesized. If the inoculum size (N_0) is greater than N', then the critical time T_0 is reduced below L. Inhibition zones are still formed unless $N_0 > N''$, when T_0 and X become nil.

(6) At different temperatures, though G varies the critical population is constant. The latter is independent of inoculum size (N_0) or antibiotic concentration (m_0).

(7) Critical population sets a limit to effective antibiotic therapy when the antibiotic has to reach the infected site by diffusion. Early therapy is essential for success, when the bacterial population is not being controlled by other defences.

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The Pattern of Growth and Flagellar Development in Motile Gram-positive Cocci

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SUMMARY: By the use of a staining technique which enables the flagella and cell walls to be demonstrated simultaneously, the distribution of flagella on the component cells of motile Gram-positive cocci has been examined. By comparison of these preparations with others made by the technique of Pennington (1950), which indicates the physiological age of cells according to the resistance of their basophilic elements to the action of formaldehyde, it is apparent that such multicellular cocci divide in the manner already suggested for rod-shaped bacteria, not by simple fission but by a process analogous to budding, so that different cells in a group are at different physiological ages or stages of development.

The proposition that bacteria grow from one or both poles and divide by a process which resembles budding was advanced by Bisset (1951) and confirmed experimentally by Bisset & Pease (1957). The main evidence is the arrangement of the flagella in growing and dividing cells, which is such as to suggest that the mother-cell retains most of its original cell wall and mature flagella, whereas the bud has a newly-grown cell wall with flagella in an early stage of development. It is of obvious interest to determine the arrangement of flagella upon dividing cells of motile cocci, but this is a task of greater complexity than the work with rod-shaped organisms, since it entails the simultaneous demonstration of the flagella and of the pattern of cross-walls dividing the cells of each group. The method to be described was eventually devised for this purpose, and it is remarkable that, although arrived at independently after prolonged trial and error, it proves to have some points in common with the technique of Shunk (1920), which was also claimed to show cell walls and flagella simultaneously, although no examples were published, and it is apparent that only the external wall was implied. The present method is less complicated and considerably less drastic.

In addition to the studies on flagellar pattern already described, a similar conclusion with respect to the division of bacteria into mother and daughter cells was reached by Pennington (1950) who showed that, after treatment with neutral formaldehyde, bacteria in young cultures retained their basophilia, whereas in older cultures they lost it. In rapidly growing cultures, recently divided pairs showed usually one ‘old’ and one ‘young’ cell. This method has now been applied to the motile cocci and provides evidence in support of the conclusions drawn from the observations on flagellar pattern. For purposes of comparison, some observations are included of the effect of this technique upon multicellular rod-shaped bacteria.

METHODS

Six strains of motile Gram-positive cocci were examined. These included a stock culture of *Sarcina ureae*, two similar saprophytes isolated by Dr B. A. D. Stocker and Mr E. B. Pike, and three organisms isolated from fish by Mr P. R. Hayes, to all of whom we are grateful. All grew at room temperature on nutrient agar. Actively motile cultures were examined at various ages from 8 to 24 hr. Suspensions of organisms were made in distilled water containing 0·5% (w/v) formaldehyde, and air-dried smears were mordanted for at least 18 hr. at 20–25° in a solution of 7·5% (w/v) tannic acid + 1% (w/v) ferric chloride in water, washed and mordanted for a further 5 min. in the same solution, with the addition of 1·5% (v/v) of a second solution containing 10% (v/v) aniline + 40% (v/v) ethanol, in water. After washing, the preparation was stained in 0·5% (w/v) crystal violet for *c.* 3 min., and allowed to dry without heating.

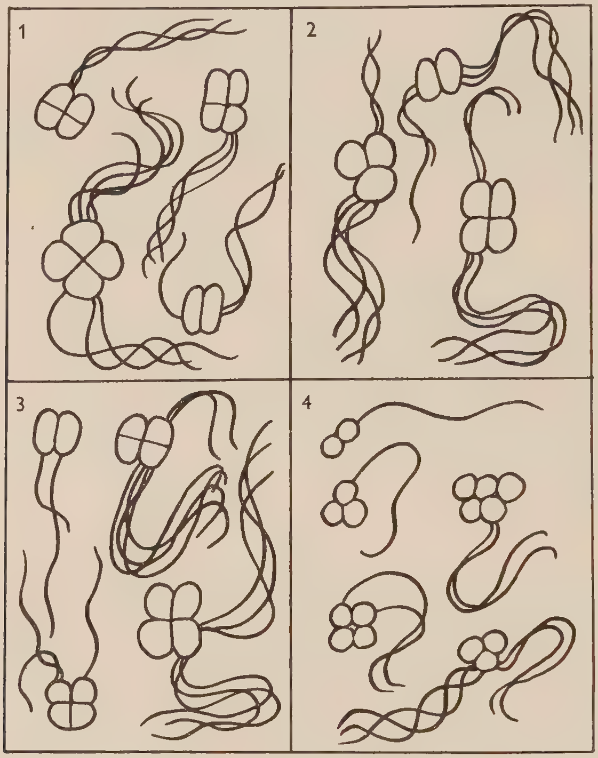
Preparations of these cocci, of one newly isolated strain of *Bacillus cereus* and one stock culture of a *Jensenia* sp., were also examined by the technique of Pennington (1950) at ages of culture from 4 hr. to 7 days. The method used was exactly as described by Pennington, with the exception that comparisons were made between preparations stained for longer and shorter times, in order to achieve different intensities of coloration. The purpose of this was to differentiate, not only between complete bacilli or cocci in their degree of loss of basophilia, but also between individual cells of a single organism, as illustrated in the figures of Pl. 2.

RESULTS

The results of the first method are shown in Pl. 1, as applied to a large saprophytic coccus of the *Sarcina ureae* type. Figures 1–3 are all printed from the same negative. The opacity of the flagella was much less than that of the cells, and Pl. 1, fig. 3, was obtained by screening the latter with a semi-opaque mask. However, the best demonstration of the finer cytological details can only be achieved by the use of separate prints. No difficulty was experienced in observing both the flagella and the cell walls simultaneously in the microscope.

The distribution of the flagella upon the cells of each group is seen to be uneven. Some have several flagella and the majority have none. Plate 1, figs. 4–7, show examples of tetrads with typically uneven distribution of flagella. All these photographs have been printed to demonstrate the flagella in this instance, but their cellular pattern can readily be understood by comparison with Pl. 1, fig. 2. The drawings in Figs. 1–4 were made from similar preparations. Figures 1–3 are different strains of the *Sarcina ureae* type; Fig. 4 is a small coccus from fish. All show the same characteristic arrangement of flagella upon the cells.

Pennington's method, as applied to cocci in very young cultures, showed groups of cells all of which took the stain well, although usually one pair in each tetrad was more basophilic than the other (Pl. 2, fig. 8). In cultures several days old most of the cells lost their basophilia, although there was again a tendency for one pair in each tetrad to stain more deeply than the other (Pl. 2, fig. 9). In actively growing cultures, (12–48 hr. old) the pattern was very strongly marked. Very often one or two cells in each group were strongly basophilic (i.e. 'young') by comparison with the remainder (Pl. 2, fig. 10). This pattern was found also in septate bacilli, where one half of a bicellular rod, or the terminal cell in a filament, was much less darkly stained than the remainder (Pl. 2, figs. 11–13).



Figs. 1–3. Drawings of three strains of large cocci of the *Sarcina ureae* type, showing distribution of flagella. Fig. 4. Drawings of a strain of coccus from fish, showing distribution of flagella.

DISCUSSION

The pattern of flagella upon the cells of motile cocci is in accordance with the theory, previously advanced for rod-shaped bacteria, that cell division is not equational, but is by a process analogous to budding. Thus the mother-cell retains the major part of the original cell wall and flagella (Bisset, 1951). In the case of the cocci examined, the lag between cell division and the development of flagella is so long that the majority of cells in a growing culture have none. Confirmation of this theory is obtainable from the study of groups of dividing cells stained by Pennington's method. Here what appear to be physiologically younger cells are more basophilic after treatment with form-

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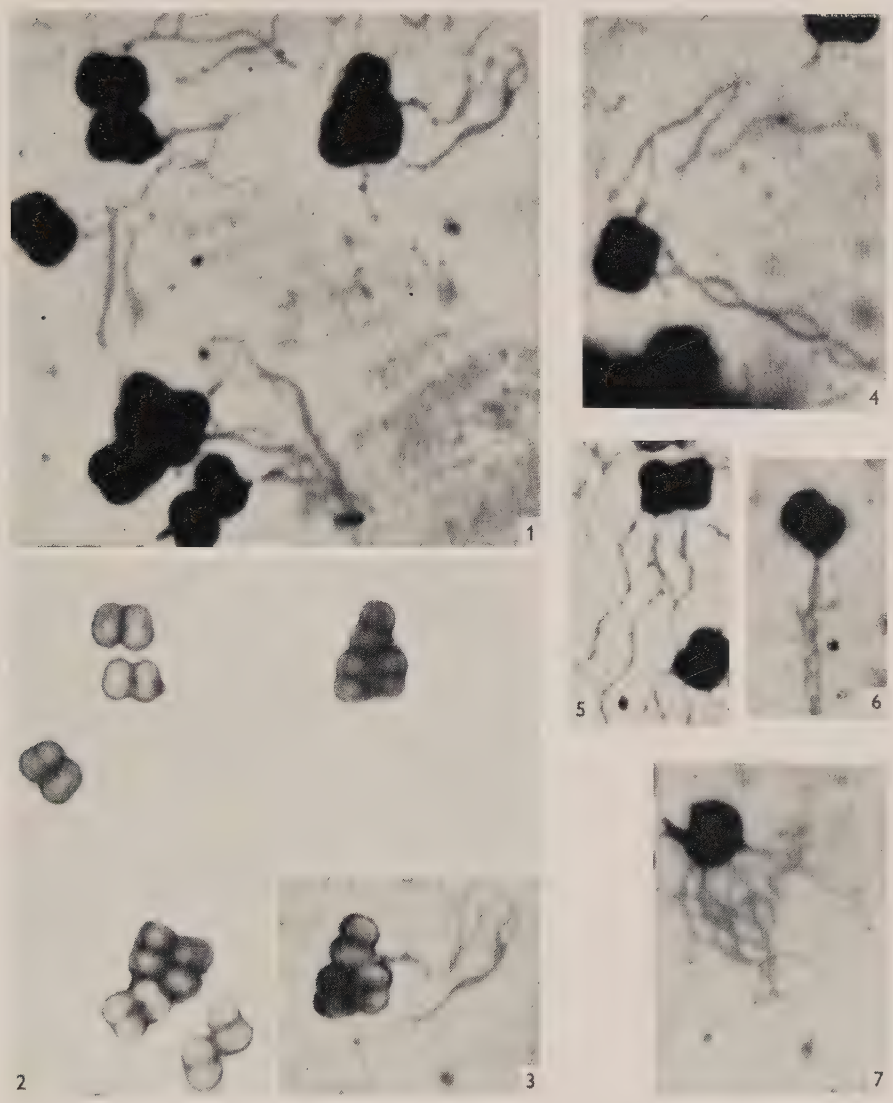

C. M. F. Hale and K. A. Bisset—Flagellar development in motile Gram-positive cocci. Plate 1

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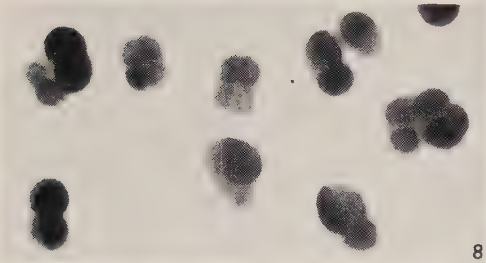
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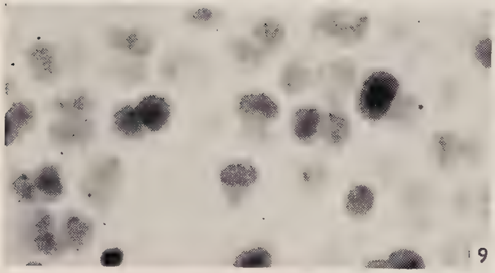
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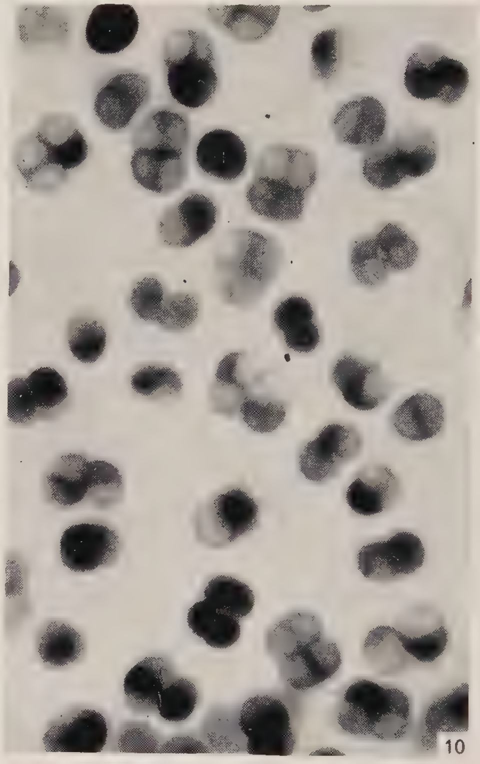
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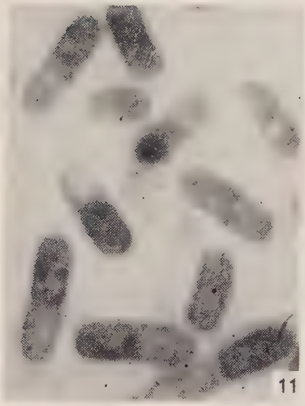
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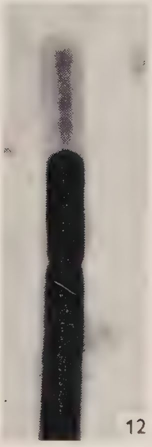
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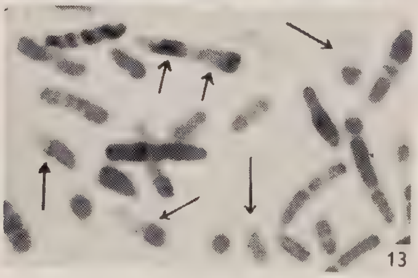
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C. M. F. HALE AND K. A. BISSET—FLAGELLAR DEVELOPMENT IN MOTILE GRAM-POSITIVE COCCI. PLATE 2 aldehyde than are their older neighbours. The arrangement of such groups of cells indicates that not all are of the same physiological age, and that the younger must be regarded as having budded from the older. The pattern in septate bacilli is comparable, and confirms this general theory of bacterial growth.

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EXPLANATION OF PLATES

Plate 1

All figures are photomicrographs of a large coccus of *Sarcina ureae* type, stained to demonstrate flagella and cell walls simultaneously, × 2500.

Figs. 1–3. All these prints are made from the same plate; fig. 1 to show the flagella; fig. 2, less deeply printed, to show the cell walls; fig. 3, printed with an opaque mask over the cells, to demonstrate both flagella and cell walls as well as the method permits.

Figs. 4–7. Tetrads showing typically uneven distribution of the flagella, printed to show the latter only. Cell walls are as in comparable groupings in fig. 2.

Plate 2

Figs. 8–10. The same coccus as in Pl. 1, stained by Pennington's method, at different ages, 4 hr., 7 days and 18 hr., respectively, showing loss of basophilia with age, and unequal distribution of 'young' cells in actively growing cultures (fig. 9). × 3000.

Figs. 11, 12. *Bacillus cereus*, 20 hr., showing differential basophilia in bacilli and in a filament. × 3000.

Fig. 13. *Jensenia* sp., 20 hr., showing differential basophilia in septate rods (arrows). × 3000.

(*Received* 10 *December* 1957)

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Adaptive Patterns in the Bacterial Oxidation of 2:4-Dichloro- and 4-Chloro-2-methyl-phenoxyacetic Acid

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SUMMARY: Organisms of a strain of *Flavobacterium peregrinum* and an *Achromobacter* strain, capable of decomposing 2:4-dichlorophenoxyacetic acid (2:4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA) respectively, have been obtained by growing cultures on peptone agar plates containing either 2:4-D or MCPA. Glucose did not prevent adaptation. Organisms of the *F. peregrinum* strain were adapted to oxidize 2:4-D when grown on peptone agar containing either MCPA or 2-chloro-4-methylphenoxyacetic acid; they did not decompose MCPA. The effects of some related compounds on adaptation to 2:4-D by these bacteria were examined. Growth of the *Achromobacter* strain on peptone agar containing either 2:4-dichlorophenol or 5-chloro-2-cresol gave organisms which were adapted to oxidize both 2:4-D and MCPA as well as the inducing compound.

Evidence was obtained by Steenson & Walker (1956, 1957) that the oxidation of 2:4-dichlorophenoxyacetic acid (2:4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA) by two different soil bacteria was effected by adaptive enzyme systems and that 2:4-dichlorophenol and 5-chloro-2-cresol were intermediates in the dissimilation of 2:4-D and MCPA respectively. Organisms of *Flavobacterium peregrinum* n.sp. (Stapp & Spicher, 1954) grown on 2:4-D could oxidize 2:4-D, 4-bromo-2-chloro- and 2:4-dibromophenoxyacetic acid and the corresponding phenols from which these acids are derived, but none of the isomeric dichlorophenoxyacetic acids. The power of an *Achromobacter* sp. to oxidize MCPA became decreased by repeated subculture in media containing 2:4-D in place of MCPA, but was restored when the organism was again grown in MCPA-containing media. On the other hand, MCPA-grown organisms of this strain could oxidize both MCPA, 2:4-D and the corresponding phenols, 5-chloro-2-cresol and 2:4-dichlorophenol.

Walker & Newman (1956) described the isolation of strains of a soil micro-organism, tentatively identified as a *Mycoplana* sp., which decomposed 2:4-D and the 2:4-D-grown organisms also oxidized 2:4-dichlorophenol. Oxidation of 2:4-dichlorophenol by a 2:4-D-decomposing *Corynebacterium* sp. has been reported by Rogoff & Reid (1956) and Bell (1957) obtained a similar finding with an *Achromobacter* sp. Earlier work by Audus (1951) with soil-enrichment cultures in a percolator had shown that there was some adaptation between MCPA and 2:4-D. Hitherto, most workers have used culture media in which 2:4-D or MCPA was the sole carbon source, with perhaps supplementary amounts of yeast or soil extracts as sources of growth factors. Indeed, Audus (1951) found that all adaptation to oxidize 2:4-D was lost if he grew his strain of *Bacterium globiforme* on media containing sugars, but presumably not 2:4-D. We have found that adapted organisms may be obtained by growth of our two bacterial strains on peptone agar or peptone glucose agar in the presence of moderate amounts of 2:4-D or MCPA. The effect on adaptive enzyme formation of a number of related aromatic compounds has been studied therefore by incorporating them at suitable concentrations in peptone media for the growth of the bacteria.

METHODS

Micro-organisms. A strain of *Flavobacterium peregrinum*, n.sp. (Stapp & Spicher) and an *Achromobacter* strain, both of which were isolated from Rothamsted soil and described by us (Steenson & Walker, 1956) previously, were used.

Media and cultural conditions. Agar plates were poured from a sterilized solution of peptone (0·2–1·0 g.), 2:4-D or MCPA (5–50 mg.) and agar (2·0 g.) in distilled water (100 ml.) adjusted to pH 7·3 by adding aqueous sodium carbonate solution. As necessary, other compounds were used in place of 2:4-D. All cultures were incubated at 25° for 2–4 days.

Manometeric methods. Organisms were washed in 0·02 M-phosphate buffer solution (pH = 6·98) and resuspended in the same buffer solution. The total nitrogen content of the suspension was determined by the usual micro-Kjeldahl procedure. Oxygen uptake by 1 ml. cell suspension (containing usually 0·15–0·4 mg. total N) was measured in Warburg manometers at 30°. The main cup of the Warburg vessel contained the suspension of organisms and enough 0·02 M-phosphate buffer solution to give a total volume of 3 ml.; the substrates were added from the side bulb, after equilibration. The centre cup contained 0·2 ml. 20 % (w/v) aqueous potassium hydroxide and the gas phase was air.

Reference compounds. 2:3-, 2:5- and 3:5-Dichloro-phenoxyacetic acids and 2:4-difluorophenoxyacetic acid were given to us by Professor R. L. Wain, F.R.S. 2-Methyl-, 4-methyl- and 2:4-dimethyl-phenoxyacetic acids were prepared by reacting the sodium salts of the corresponding phenols with sodium monochloroacetate. 2-Chloro-4-methylphenoxyacetic acid was synthesized for us by Mr J. Heidemeyer. Other compounds were obtained commercially.

RESULTS

The effect of peptone and glucose on adaptation

Cultures of the *Achromobacter* strain were grown on peptone (0·5 %) glucose (0·5 %) agar plates for 3 days with and without 2:4-D (0·05 %). The rates of oxygen uptake by the harvested organisms were measured in the presence of 2:4-D or 2:4-dichlorophenol as substrate. The results, given in Table 1, showed that the organisms were adapted to oxidize the substrates only after growth in the presence of 2:4-D; neither peptone nor glucose prevented adaptation from taking place.

In another experiment, organisms of *Flavobacterium peregrinum* adapted to oxidize 2:4-D were obtained by growth in a peptone (0·5 %) agar medium containing 2:4-D (0·05 %). When grown on peptone agar alone, the organisms obtained were not adapted to oxidize 2:4-D (Table 2).

Table 1. *Respiration of organisms of* Flavobacterium peregrinum *grown on different media, in the presence of various substrates*

Oxygen uptakes were determined in Warburg manometers at 30° as described in the text. q_{O_2} represents μl. of O_2/mg. cell N/hr. The endogenous respiratory rate was not subtracted in calculating the q_{O_2} values in this table.

Medium	Substrate	q_{O_2}
Peptone	None	40
	2:4-D	50
Peptone + 2:4-D	None	40
	2:4-D	600
Peptone + MCPA	None	125
	2:4-D	1050
	MCPA	275
	5-Chloro-2-cresol	400
Peptone + 2-chloro-4-methylphenoxyacetic acid	None	100
	2:4-D	260
	MCPA	125
	2-Chloro-4-methylphenoxyacetic acid	195

Table 2. *Respiration of* Achromobacter *organisms, grown on different media, in the presence of various substrates*

Oxygen uptakes were determined in Warburg manometers at 30° as described in the text. q_{O_2} represents μl. of O_2/mg. cell N/hr. The endogenous respiratory rate was not subtracted in calculating the q_{O_2} values in this table.

Medium	Substrate	q_{O_2}
Peptone + glucose	None	214
	2:4-D	285
	2:4-Dichlorophenol	190
Peptone + glucose + 2:4-D	None	220
	2:4-D	860
	2:4-Dichlorophenol	810
Peptone + 2:4-dichlorophenol	None	207
	2:4-D	1070
	MCPA	570
	2:4-Dichlorophenol	750
	5-Chloro-2-cresol	336
Peptone + 5-chloro-2-cresol	None	86
	2:4-D	666
	MCPA	566
	2:4-Dichlorophenol	140
	5-Chloro-2-cresol	233

The effect of MCPA on Flavobacterium peregrinum

Cultures of *Flavobacterium peregrinum* were grown on 0·5 % peptone agar containing MCPA (0·05 %) for 3 days. These organisms were not adapted to oxidize MCPA but gave a considerable oxygen uptake in the presence of

2:4-D or 5-chloro-2-cresol. Thus, although MCPA itself is not metabolized by this organism, it can induce in *F. peregrinum* the enzymes necessary for the oxidation of 2:4-D.

The effect of related compounds on Flavobacterium peregrinum

The activity of organisms grown on peptone agar with a series of substituted phenoxyacetic acids was examined.

2-Chloro-4-methylphenoxyacetic acid behaved like the isomeric MCPA in that it induced the adaptation of the organisms to oxidize 2:4-D (Table 2). The organisms also oxidized the inducing compounds to some extent.

None of the following substances caused any adaptation to 2:4-D oxidation: phenoxyacetic acid, 2-methyl-, 4-methyl- and 2:4-dimethyl-phenoxyacetic acid; 2-chloro-, 4-chloro-, 2:3-, 2:5- and 3:5-dichloro-phenoxyacetic acid; 2:4-difluorophenoxyacetic acid.

The effect of 2:4-dichlorophenol on the Achromobacter *strain*

Cultures of the *Achromobacter* strain were grown on peptone (0·2 %) agar medium containing 2:4-dichlorophenol (0·004 %) for 3–4 days. The harvested organisms were adapted to oxidize 2:4-D, 2:4-dichlorophenol, 4-chloro-catechol, and, at a slower rate MCPA and 5-chloro-2-cresol. 2:4-Dichlorophenol was oxidized only at low concentrations, viz. 1 μmole/3 ml., and twice this concentration was inhibitory.

The effect of 5-chloro-2-cresol on the Achromobacter *strain*

Similar experiments to the above were carried out in which cultures were grown on peptone (0·2 %) agar containing 5-chloro-2-cresol (0·004 %). 2:4-Dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid were oxidized most rapidly, 5-chloro-2-cresol was oxidized more slowly and 2:4-dichlorophenol scarcely at all.

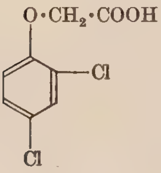
The effect of 2:4-dichlorophenol on Flavobacterium peregrinum

It was found that 2:4-dichlorophenol was rather inhibitory to the growth of this strain even at a concentration of 50 parts per million, and it was difficult to obtain enough organisms in 3–4 days for manometry. However, in one experiment, organisms grown on peptone (0·25 %) agar containing 2:4-dichlorophenol (32 parts per million), showed a moderate rate of oxygen uptake with 2:4-dichlorophenol (1 μmole/3 ml.), a slower one with 5-chloro-2-cresol and very feeble oxidation of 2:4-D.

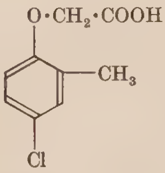
DISCUSSION

The fact that organisms adapted to oxidize 2:4-D or MCPA could be obtained from cultures grown on peptone media, provided that small concentrations of 2:4-D or MCPA were also present suggested that the effect of other compounds might be tested similarly. It seemed that growth in the presence of a suitable inducing compound was all that was necessary to cause the formation of the adaptive enzyme systems involved and the inducing compound need not be the only substance metabolized by the bacteria during their growth. It is interesting that both 4-chloro-2-methylphenoxyacetic acid (MCPA) and the isomeric 2-chloro-4-methylphenoxyacetic acid could also induce in *Flavobacterium peregrinum* the adaptive enzymes for oxidizing 2:4-D. This organism has not been shown to decompose MCPA in soil cultures whereas it readily attacks 2:4-D.

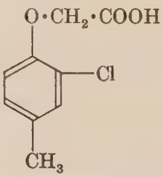
The effect of 2:4-dichlorophenol or 5-chloro-2-cresol in adapting organisms of the *Achromobacter* strain to oxidize 2:4-D and MCPA is not contrary to the theory of simultaneous adaptation outlined by Stanier (1947). It was pointed out by Stanier that in a complex dissimilation an enzyme may act at more than one stage in the process. Hence when two intermediates, say D and E, are separated by one enzymic step, the possibility exists that the enzyme catalysing that particular step (D → E) may also function later on in the oxidation of E, and that growth on E will also adapt the cells completely for the attack on D. If two intermediates, say B and E, are separated by several intervening steps (B → C → D → E), growth on E is not likely to produce cells completely adapted for the oxidation of B. In the present case, the above phenols may be assumed to be the first oxidation products of 2:4-D and MCPA respectively and can apparently induce adaptation of bacteria to oxidize their immediate precursors.



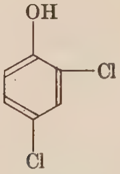
2:4-D



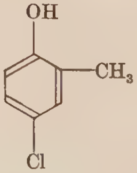
MCPA



2-Chloro-4-methyl-phenoxyacetic acid



2:4-Dichlorophenol



5-Chloro-2-cresol

In all these cases, the inducing compounds are 2:4-disubstituted phenols or phenoxyacetic acids, and it is clear that position isomerism is the dominant factor determining the specificity of the adaptation.

We wish to thank Dr H. G. Thornton, For.Sec.R.S., for his interest in this work, and Professor R. L. Wain, F.R.S., for gifts of certain compounds.

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(*Received* 18 *December* 1957)

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The Effect of Ferrous Ions on the Formation of Toxin and Porphyrin by a Strain of *Corynebacterium diphtheriae*

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SUMMARY: The effect of the iron concentration in the medium on the concentration of toxin and coproporphyrin in the culture filtrate of a strain of *Corynebacterium diphtheriae* has been found to differ quantitatively from that previously reported in the case of the P.W. 8 strain. Only 50–60 % of the iron present in the medium, at all concentrations tested, was recoverable from the organisms. Of the total iron present in the medium, only 9 % was found to be present in the organisms as a haem complex capable of measurement as its pyridine haemochromogen. On the basis of their iron contents protohaemin and iron coproporphyrin III are less active than ferrous sulphate in their effect on toxin yield. These findings do not support the hypothesis that diphtheria toxin is the protein moiety of the cytochrome *b* of the cell.

Campbell-Smith (1930) in England, and Coulter & Stone (1930) in America reported the occurrence of a porphyrin-like pigment in diphtherial culture filtrates. It was noticed that when filtrates were stored in the light, the toxin and the pigment disappeared at the same rate, and attempts were made to correlate the formation of the two products during growth. The impression that the toxin and pigment were identical was dispelled by the demonstration (Wadsworth, Crowe & Smith, 1935; Wheeler & Crowe, 1936) that the pigment could be completely removed by charcoal treatment without decreasing the toxin titre. Pappenheimer & Johnson (1936) found that there was an optimum concentration of iron in the medium for diphtheria toxin production. Any increase in the iron concentration of medium above this concentration led to a decrease in the toxin titre while causing no decrease in growth of the organism. Re-investigating these two phenomena quantitatively, Pappenheimer (1947) found that for every four atoms of iron added to the culture medium above that optimal for toxin production, four molecules of porphyrin and one molecule of toxin failed to appear in the culture filtrate. All the iron added to the medium over the range which inhibited toxin formation could be recovered from the bacterial cells. It was suggested that this evidence indicated that the toxin was the protein moiety of a respiratory enzyme of the organism. Pappenheimer & Hendee (1947) investigated the formation of catalase, cytochromes *b* and *c* and cytochrome oxidase by two strains of *Corynebacterium diphtheriae*, and found that cytochrome *b* was the main iron prophyrin-containing pigment of the organism; they suggested that cytochrome *b* might be composed of iron, porphyrin and toxin.

Pappenheimer (1947) had found that the diphtheria porphyrin showed absorption curves almost identical with those of haematoporphyrin when examined in N-HCl between 380 and 620 mμ. Gray & Holt (1948) subsequently found that the main porphyrin component of diphtheria culture filtrates was in fact coproporphyrin III.

Pappenheimer investigated the changes in the succinoxidase activity of toxaemic guinea-pigs. Since cytochrome *b* appears to be the rate-controlling compound in this enzyme system it might be expected that the effect of diphtheria toxin, if related to cytochrome *b*, would be to decrease the succinoxidase activity of the tissues. These observations were inconclusive, and further investigations into the action of the toxin were undertaken, using the Cecropia silk-worm. The injection of less than 1 μg. of diphtherial toxin into mature caterpillars, prepupae and developing adults caused a cessation of development and death in 1–5 weeks (Pappenheimer & Williams, 1952). Dormant pupae, on the other hand, survived over 4 weeks after the injection of 70 μg. toxin, though 1/100 of this dose prevented the pupae from initiating adult development. Maximum sensitivity to the toxin was found to be characteristic of those developmental stages which showed a cytochrome system; even within the diapausing pupae the toxin failed to affect heart muscle, from which the normal cytochrome sytem was absent, while it caused obvious degeneration of the intersegmental muscles which possessed an intact cytochrome system. This evidence strongly supported the thesis that the toxin acted on one or other components of the Cecropia cytochrome system. The arrest of adult development by toxin as opposed to the long latent period required before degenerative changes became evident was considered to indicate an interference with the synthesis of a component rather than with its function.

The quantitative investigations of Pappenheimer & Hendee were carried out with the 'Toronto' P.W. 8 strain of *Corynebacterium diphtheriae*. The G/12/6 strain, used in this laboratory for routine toxin production, was known to differ in several important respects from the 'Toronto' strain. In particular, the G 12/6 strain produced higher toxin titres on a low nitrogen medium. It was therefore considered of interest to investigate whether the quantitative relationship of iron, toxin and porphyrin observed with the 'Toronto' strain also held for the G 12/6 strain. This paper describes the findings of this investigation, a preliminary note of which has already been published (Clarke & Clarke, 1953).

METHODS

Organism. The strain used throughout was the G 12/6 strain of *Corynebacterium diphtheriae* used for routine toxin production in this laboratory.

Medium and conditions of growth. The medium used was a modification of that used by Holt (1948): acid hydrolysate of casein to 0·1 % (w/v) N; cystine, 0·2 g.; nicotinic acid, 9 mg.; β-alanine, 2·3 mg.; pimelic acid, 0·15 mg.; $CuSO_4.5H_2O$, 1 mg; $ZnSO_4.7H_2O$, 0·8 mg.; $MnCl_2.4H_2O$, 0·3 mg.; maltose, 12·5 g.; lactic acid (A.R.), 3 ml.; $CaCl_2$ (A.R. dried), 0·125 g.; distilled water to 1 l.; pH adjusted to 6·8.

The casein hydrolysate, maltose and cystine were 'deferrated' as described by Holt (1948) and the maltose was autoclaved in the medium except in large-scale cultures, when it was autoclaved separately and added to medium autoclaved at pH 7·6. Cultures, unless otherwise stated, were grown in 30 ml. volumes of medium which had been autoclaved at 15 lb./sq.in. (115°) for 15 min. in 100 ml. Pyrex Erlenmeyer flasks. After inoculation by a loop of platinum wire from a 24 hr. pellicle, the cultures were incubated unshaken at 34°.

Toxin + toxoid titres. Toxin + toxoid was estimated by flocculation test (Ramon, 1922). When the value was < 25 Lf units/ml. the filtrate was blended with a toxin of known titre before testing. In the sequel 'toxin' refers to toxin + toxoid determined in this way.

Amount of growth of organism. Bacterial growth was determined on saline-washed organisms by determination of total-N by the micro-Kjeldahl method (Markham, 1942).

Protein determination. The protein content of culture filtrates was determined by precipitation with 5% (w/v) trichloroacetic acid and determination of total-N on the precipitate, after it had been washed with trichloroacetic acid.

Coproporphyrin determination. Coproporphyrin was extracted from culture filtrates by a modification of the method used by Pappenheimer (1947), and was determined spectrophotometrically. To 5 ml. of toxin filtrate in a beaker was added 5 ml. 10 N-HCl. The beaker was placed on a boiling water-bath in diffuse light for 10 min. and then allowed to cool. To the beaker was then added 2·5 ml. glacial acetic acid, and the pH value adjusted to 3·6 with 50% (w/v) NaOH. The solution was allowed to cool and then transferred quantitatively to a 50 ml. separating funnel. An equal volume of ether was added and the funnel shaken gently for 1 min. The layers were allowed to separate completely before the water layer was run back into the beaker. The ether layer was washed twice with an equal volume of 0·1% (w/v) sodium acetate adjusted to pH 3·6 with N-HCl, and the porphyrin then extracted by three volumes of 0·1 N-HCl. The filtrate was returned to the funnel and the extraction process repeated. The HCl extracts were made up to 50 ml. and compared with a standard solution of coproporphyrin III in 0·1 N-HCl at the Soret peak (402 mμ.).

Iron determination. The iron concentration in the organisms was determined by the *o*-phenanthroline method (Saywell & Cunningham, 1937; Fortune & Mellon, 1938). The saline-washed organisms were dried at 160° in Pyrex test tubes and heated gently with 1 ml. of combustion mixture (redistilled HNO_3, 80 vol.: $HClO_4$ (A.R.), 5 vol.: H_2SO_4 (A.R.), 3 vol.) until all solid matter had disintegrated. The tubes were then kept in a water bath at *c.* 80° for 3 hr. and finally heated slowly until colourless. After cooling, 1 ml. of distilled water was added to each tube, followed by 0·4 ml. of 10% (w/v) hydroxylamine hydrochloride (A.R.) and 0·25 ml. of a 1% (w/v) solution of *o*-phenanthroline (A.R.). The reaction was adjusted to pH 6·0 ± 1·0 with NH_4OH (A.R.) by using a micropipette and an external indicator paper. The tubes were kept at 20–25° for 2 hr., and then made up to 20 ml. The iron content was determined by comparison with a standard, using a blue-green filter in a Hilger spectrophotometer.

Metalloporphyrin. The iron complex of coproporphyrin III was obtained by saponification of its tetramethyl ester prepared by the method used by Fischer & Orth (1937) for the I tetramethyl ester.

Haem determination. The haem content of organisms was determined by the pyridine haemochromogen method of Pappenheimer & Hendee (1949), or by extraction by the method of Rawlinson & Hale (1949) as indicated.

RESULTS

Toxin and porphyrin concentration

In order to relate toxin and porphyrin concentrations to the amount of iron added to the medium it was necessary to determine after what period of incubation these concentrations were maximum, at which concentrations only could legitimate comparison be made. Fig. 1 shows the results of growth at optimum iron concentration (0·2 μg./ml.) and optimum iron concentration +0·2 μg. Fe/ml. After 7 days of incubation the bacterial-N was beginning to decline, but the toxin and porphyrin concentrations were approximately maximum. The graph of pH value with time shows that the pH values varied between 6·6 and 7·9, between which limits toxin and coproporphyrin were found to be stable under the conditions of growth. In some experiments the pH value fell as low as 6·2 for a period of 24 hr., but at this pH value there was no decrease in toxin titre during 48 hr.

Effect of iron on toxin and porphyrin formation

The term 'formation' is used to mean the appearance of toxin and porphyrin in the culture filtrate during the growth of the diphtheria bacillus. Whether or not this is a true index of the formation of these substances by the cells depends on their existence in the cells, before their appearance in the filtrate, in a concentration in excess of that found in the filtrate (Raynaud *et al.* 1954). It is known that both toxin and coproporphyrin, once released, remain stable in the filtrate for a considerable time, and the concentrations determined are thus a true indication of the amounts released from cells up to the time of harvesting.

The effect of the addition of ferrous sulphate, in concentrations up to 0·86 μg. iron/ml. culture medium, on the toxin and coproporphyrin release, is shown in Table 1. The cultures were harvested after 7days of incubation at 34° when the toxin and coproporphyrin yields were at a maximum. It can be seen that the increase in iron concentration had little effect on the growth of the culture as indicated by the bacterial-N determinations on washed organisms. The relationship between iron added and the toxin and porphyrin failing to appear in the culture filtrate is shown in Fig. 2. These figures, expressed in molar concentrations with the molecular weight of diphtheria toxin being taken as 72,000 (Pappenheimer, 1942), indicate that approximately 1 molecule of toxin and 2 molecules of porphyrin failed to appear in the culture filtrate for every 2 atoms of iron added to the medium in excess of that required for maximum toxin formation. Iron determinations on washed organisms,

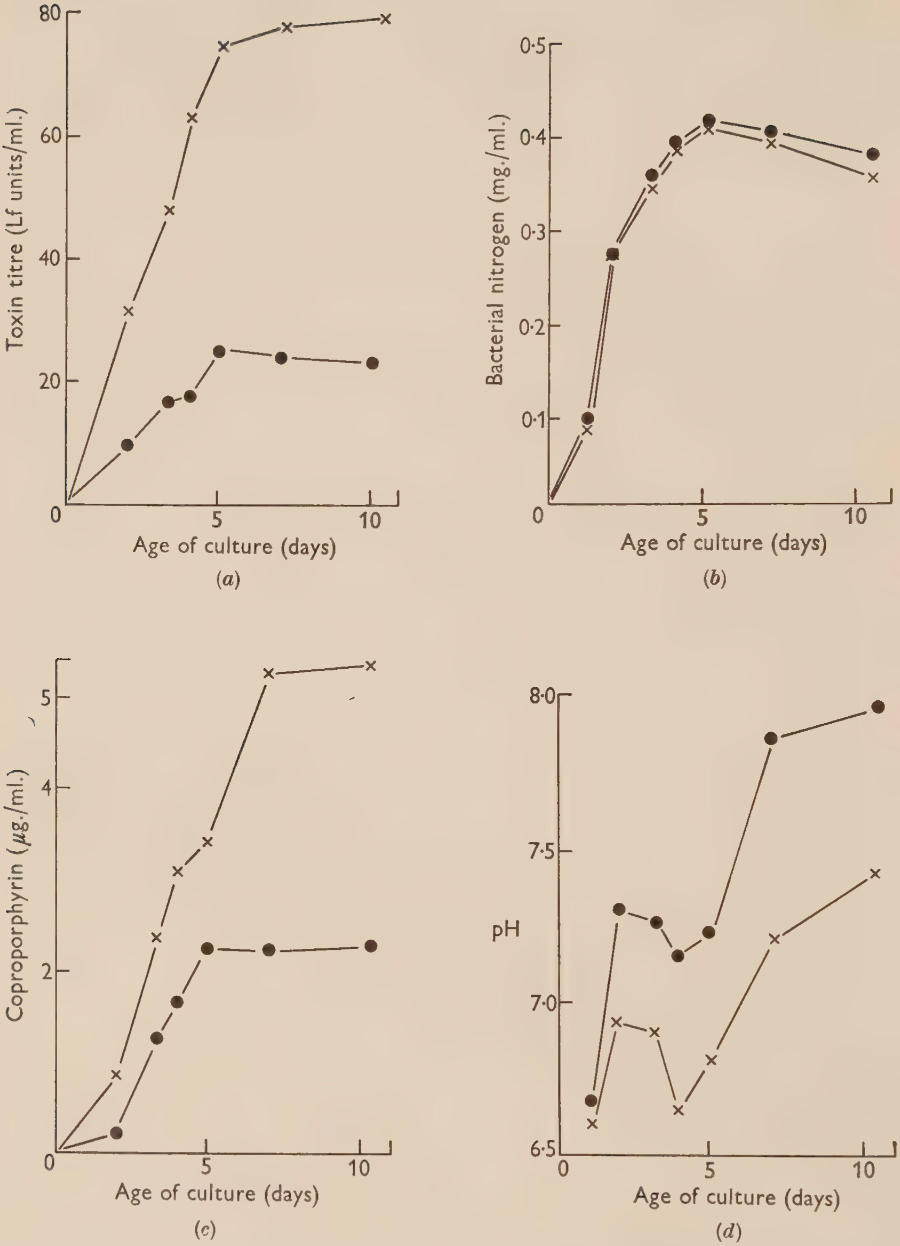

Fig. 1. Cultures of *C. diphtheriae* grown at 34° for 10 days containing: ×—×, 0·22 μg. of iron per ml.; ●—●, 0·42 μg. of iron per ml. (*a*) Toxin titre of culture filtrate; (*b*) bacterial nitrogen; (*c*) coproporphyrin concentration in culture filtrate; (*d*) pH.

Table 1. *The effect of increasing iron concentrations on the toxin titre and coproporphyrin concentration in filtrates of cultures of* Corynebacterium diphtheriae *strain G* 12/6

30 ml. cultures in 100 ml. conical flasks (Pyrex) were incubated for 7 days at 34°. Organisms were centrifuged down and washed twice with 0·85 % (w/v) NaCl solution. All values are averages of duplicate cultures.

Iron concn. in medium (μg./ml.)	Toxin titre (Lf/ml.)	Bacterial-N (μg./ml.)	Coproporphyrin (μg./ml.)	Bacterial-Fe (μg./ml.)	pH value
0·20	92	350	5·3	0·11	6·71
0·24	85	355	4·8	0·13	6·74
0·28	75	365	4·3	0·14	6·76
0·32	65	370	4·0	0·16	6·68
0·40	45	340	3·5	0·23	6·71
0·56	10	350	1·15	0·31	6·66
0·86	3	365	0·40	0·44	6·71

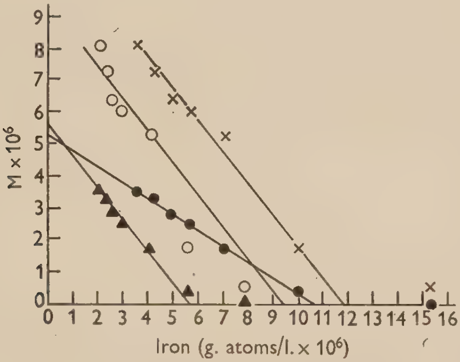

Fig. 2. Toxin and coproporphyrin concentrations related to iron in medium and cells. ●—●, Toxin, and ×—×, coproporphyrin, related to iron concentration in medium. ▲—▲, Toxin and ○—○, coproporphyrin related to iron in washed cells. Cultures of *C. diphtheriae* grown at 34° for 7 days. The lines represent ratios of 1:1 and 2:1 respectively.

however, indicated that little over half the iron present in the medium was recoverable from the organisms, and the relationship should logically be based on these figures if, as is postulated, these substances exist in combination within the organisms. On this basis (Fig. 2) there is reasonable agreement with the relationship 1 atom of iron:2 molecules coproporphyrin:1 molecule toxin, except at higher iron concentrations, where the effect of iron is less marked.

The haem content of organisms

It is not satisfactory, in calculating the quantitative relationship between iron, toxin and porphyrin, to use the figures for the iron contained in the organisms, unless it can be shown that a major proportion of this iron exists or has existed in the hypothetical complex with toxin and porphyrin, namely, cytochrome *b*. It was shown by Rawlinson & Hale (1949) that cytochrome *b* is the main cytochrome component of the diphtheria bacillus. They found that 1·0–1·1 mg. protohaemin/l. culture could be extracted from the organisms.

This figure would contain the protohaemin from cytochrome *b*, but also, in all probability, some from catalase and peroxidase (if present), since it has been shown that haemin can be obtained from these enzymes, in other systems, by the HCl-acetone extraction procedure used by Rawlinson & Hale (Stern, 1936; Theorell, 1940).

Five l. medium containing an iron concentration just sufficient to depress the toxin concentration below that measurable by flocculation (3 Lf units/ml.) were inoculated with the G 12/6 strain and incubated for 7 days. The organisms were harvested by centrifugation and washed twice with 0·85% (w/v) NaCl solution. The protohaemin was then extracted by the method used by Rawlinson & Hale (1949), which consisted of defatting with ethanol and acetone, exhaustive extraction of the haemin with HCl acetone, washing the crude extract with ether, HCl and NaCl solutions, and removal of the haemin into 0·1 N-KOH. The KOH solution of crude haemin was neutralized with HCl and CO_2 bubbled through it; after being kept at 2° for 18 hr. the haemin was centrifuged down, dissolved in a pyridine + chloroform mixture, and determined spectrophotometrically (Holden, 1941). Of the total iron present in original medium (0·65 μg./ml.), haemin equivalent to 0·028 μg. iron/ml. was found in the extract in the present experiment. Thus 60% of the available iron was recoverable from the organisms, and of this only 7% was recovered as haemin. The extraction procedure was carried out quantitatively and added haemin was found to be accurately determined by this method; moreover, none was detected in the residue as pyridine haemochromogen. However, it was thought possible that a significant amount of haemin might have been adsorbed on to the bacterial residue. The proportion of haem and non-haem iron present in organisms harvested after 1–7 days of growth was therefore investigated, determining haem in cell debris after sonic disintegration (Pappenheimer & Hendee, 1949).

The organism was grown on a medium containing 0·75 μg. iron/ml., 200 ml. cultures being incubated in 1 l. bottles. Duplicate determinations of bacterial-N and iron were made on unwashed organisms from two bottles taken at intervals throughout the incubation period, and haem-iron was determined on a suspension of organisms after 30 min. distintegration in a Mullard Ultrasonic Generator E. 7590A at 20 kcyc./sec. The results (Table 2) confirm previous results in that only 66% of the iron was found in the organisms. The haem-iron content of the organisms, increasing somewhat during incubation, was never more than 14% of the iron content or 9% of the total iron concentration in medium.

Since only a small proportion of the iron present in the diphtheria bacilli could be demonstrated to be present as haem, the comparative effects of ferrous iron and haemin iron in preventing the release of toxin were investigated, and as coproporphyrin III was the main porphyrin released by the organism under conditions of iron deficiency, the effect of iron coproporphyrin III complex was also tested. Table 3 shows the effect on the toxin titre of culture filtrates at 7 days compared with a control culture which contained concentration of iron optimum for toxin production. Haemin and iron

Table 2. *The iron and haem content of organisms in cultures of* Corynebacterium diphtheriae *strain G* 12/6

Organisms were harvested at intervals from 2 × 200 ml. cultures containing 0·75 μg. iron/ml. Incubation at 34°. Duplicate determinations of bacterial-Fe and -N made on unwashed organisms.

Incubation time (hr.)	Bacterial-N (μg./ml.)	Bacterial-Fe (μg./ml.)	Bacterial-haem (μg./ml.)	Bact.-Fe / Total-Fe	Haem-Fe / Bact.-Fe
31	0·205	0·21	0·19	0·28	0·09
43	0·320	0·31	0·27	0·41	0·09
66	0·455	0·37	0·48	0·49	0·13
92	0·525	0·41	0·56	0·55	0·14
113	0·515	0·50	0·65	0·66	0·13
161	0·475	0·50	0·69	0·66	0·14

Table 3. *The relative effects of iron, iron coproporphyrin III, and protohaemin on the toxin titre of cultures of* Corynebacterium diphtheriae *strain G* 12/6

30 ml. cultures in 100 ml. conical flasks (Pyrex) were incubated for 7 days at 34°. The basal medium contained 0·21 μg. iron/ml. Metalloporphyrin solutions were sterilized separately.

Addition	Fe concentration added (μg./ml.)	Toxin titre (Lf units/ml.)
Nil	Nil	73
$FeSO_4$	0·1	49
$FeSO_4$	0·2	17
$FeSO_4$	0·3	7
Haemin	0·1	73
Haemin	0·2	63
Haemin	0·3	49
Iron coproporphyrin	0·15	64

coproporphyrin III complex both caused a decrease in toxin titre, but this decrease was less than that caused by adding ferrous iron.

DISCUSSION

The hypothesis which explains the role of diphtheria toxin in the diphtheria bacillus as that of the protein moiety of cytochrome *b*, and its toxic effects in mammals as due to interference with the synthesis of the specific mammalian cytochrome *b* is attractive, and has been supported by a quantity of largely circumstantial evidence. Pappenheimer showed that the 'Toronto' P.W.8 strain of *Corynebacterium diphtheriae* gave a close quantitative relationship between the amount of iron added to the culture medium in excess of that required for maximum toxin formation and the amounts of toxin and porphyrin which failed, under these conditions, to appear in the culture filtrate. The ratio of four atoms of iron and four molecules of porphyrin to one molecule of protein corresponds with that found in cytochrome *b*. The relationship, however, is based on the molar extinction coefficient of the porphyrin as approximating to that of haematoporphyrin, namely, $3{\cdot}46 \times 10^5$ in N-HCl, on the fact that all the iron is recoverable from the organisms, and that this iron is present in the organisms largely as cytochrome *b*.

It was shown by Gray & Holt that the porphyrin was in fact predominantly coproporphyrin III whose molar extinction coefficient in N-HCl is $5{\cdot}3 \times 10^5$. The present communication has described several respects in which the G 12/6 strain differs in behaviour from the 'Toronto' P.W.8 strain. At any concentration up to that required to decrease the toxin titre to less than 3 Lf units/ml. only 60 % of the iron present in the medium is recoverable from the organisms. The quantitative relationship between the iron, coproporphyrin and toxin is not the same as that with the 'Toronto' P.W.8 strain, nor does a constant relationship hold for all iron concentrations, since there is a decrease in the effect of iron as its concentration increases; when only bacterial iron is considered, the departure from these proportions is even greater.

If haemin, as determined by the haemochromogen method of Pappenheimer, is a reliable indication of cytochrome *b* haem, then only 9 % of the iron present in the medium initially is converted to cytochrome *b*. This means that there is no quantitative evidence for assuming that iron, porphyrin and toxin comprise diphtherial cytochrome *b*, in the G 12/6 strain. Moreover, though cytochrome *b* is undoubtedly the main iron porphyrin enzyme of the diphtheria bacillus, it is only responsible for 16 % of its iron content.

In assessing the present results it is necessary to bear in mind that the cultures on which these measurements were made represent a dynamic system. Toxin and coproporphyrin, once released into the culture medium, are stable under the conditions of growth for the period (7 days) of the observations, but it remains a possibility that diphtherial cytochrome *b* has a short life, and that more cytochrome *b* has been synthesized in the cells in the 7 days' period of growth than is determined in the final culture. There is little evidence for such a short life for respiratory enzymes however, and during this period there is insufficient lysis materially to invalidate the interpretation of the analytical results. In this connexion it is well to record that only 10 % of the porphyrin nuclei present as coproporphyrin at low iron concentrations were demonstrable as haem in conditions of iron excess; a similar situation was described by Lascelles (1956). Suspensions of *Rhodopseudomonos spheroides* converted glycine and α-oxoglutarate into porphyrins in medium of low iron concentration. When the iron content of the medium was increased, however, bacteriochlorophyll and haem were formed, which only accounted for 10 and 1 %, respectively, of the porphyrins which fail to appear in the free state. It may be said that, with the conditions of static culture used in the present investigation, there was found no quantitative evidence to support any relationship between toxin and cytochrome *b* in the G 12/6 strain of *Corynebacterium diphtheriae*.

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The Effect of Cobaltous Ions on the Formation of Toxin and Coproporphyrin by a Strain of *Corynebacterium diphtheriae*

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SUMMARY: Several cations have been shown to affect protein and porphyrin formation by the G 12/6 strain of *Corynebacterium diphtheriae*. Cobaltous ion (Co^{++}), at concentrations only slightly inhibiting growth, caused a marked inhibition of toxin formation. This effect was not produced by vitamin B_{12} or annulled by increases of cysteine or histidine concentration. Only a small proportion of added Co^{++}, at concentrations above 0·2 μg./ml., was found in washed organisms, and the effect of Co^{++} was more marked than that of Fe^{++} when based on the weight of metal in the organisms. No appreciable proportion of the Co^{++} existed as a porphyrin complex. The growth inhibition caused by 5 μg. Co^{++}/ml. was annulled by 0·6 μg. Fe^{++}/ml. Co^{++} caused no increase in the cytochrome *b* content of the organism, nor did it affect the iron concentration required for maximum toxin formation. The combined toxin-inhibitory effect of $Fe^{++}+Co^{++}$ was considerably less than the sum of their independent effects. The bearing of these findings on the thesis that diphtheria toxin is the apoenzyme of cytochrome *b* is discussed.

A concentration of 0·8 μg. ferrous ion/ml. in the culture medium almost completely prevents the appearance of toxin in culture filtrates of the G 12/6 strain of *Corynebacterium diphtheriae* (Clarke, 1958). Of 0·4–0·5 μg. iron/ml. found in the organisms only 9 % can be extracted as protohaemin from saline-washed organisms, most of which is derived from bacterial cytochrome *b* (Pappenheimer & Hendee, 1949; Rawlinson & Hale, 1949). Of the residual iron, a small fraction can be removed by extraction with citrate buffer, but the remainder is firmly bound in the organisms and only half of it is removable by extraction with HCl acetone. Since cytochrome *b* is the predominant respiratory enzyme of the *C. diphtheriae*, there is some difficulty in deciding the possible site and function of this remaining iron. Several enzyme systems known to be present in bacteria, e.g. peptidases and aldolase (Mohamed & Greenberg, 1945; Berger, Johnson & Peterson, 1938) are known to be activated by iron and in this role iron can be replaced by a number of other divalent cations. The metallic ions, however, in these enzymes are not firmly bound components, comparable with haem iron in cytochromes, and do not seem likely to remain in the organisms after washing with citrate solutions. Nevertheless, it seemed worthwhile to examine whether only iron inhibited toxin formation or whether this inhibition could be caused also by other cations. The present contribution reports the inhibition of diphtheria toxin formation by several other cations besides iron, and a detailed examination of the action of cobaltous ions; a preliminary account of this work has already appeared (Clarke & Clarke, 1953).

METHODS

The G 12/6 strain of *Corynebacterium diphtheriae*, the medium used throughout this investigation, and the methods for the determination of toxin, coproporphyrin, culture filtrate protein-N, bacterial-N, iron and haem were the same as previously described (Clarke, 1958).

Cobalt determination. The cobalt content of washed organisms and cellular fraction was determined by a modification of the method of Marston & Dewey (1940). This method, which allowed the determination of 2 μg. cobalt/10 g. plant material, involved the extraction of cobalt with diphenyl thiocarbazone to prevent the interference of the iron present in so large a quantity of material. This procedure proved unnecessary in the present work on the analysis of bacteria grown in the presence of cobalt, since the concentrations of iron and cobalt were of the same order. The cultures were centrifuged for 1 hr. at 3000 rev./min. in Pyrex test tubes (19 × 150 mm.) and the supernatant fluid was poured off and kept for toxin, coproporphyrin and protein analysis. The organisms were emulsified in 25 ml. ice-cold 1 % (w/v) NaCl warmed for 1 min. in a water bath at 45°, left at room temperature (*c* 18°) for 30 min. and recentrifuged. This washing was repeated twice more, and the organisms then dried at 150° for 1 hr. Combustion was effected in 2 ml. of a mixture of redistilled nitric acid (80 ml.) + A.R. sulphuric acid (3 ml.) + A.R. perchloric acid (5 ml.), the tubes being heated until nitric oxide was evolved; the mass of organisms was then broken up and left at room temperature for final slow combustion to near dryness the following day. When the tubes had cooled 1 ml. of 0·2 N-citric acid was added to each, and the contents transferred quantitatively to silica dishes, 1 ml. of 0·2 % nitroso-R-salt added with stirring and the reaction adjusted to pH 8·0 with buffered NaOH by using a pH meter. After well washing the electrodes, the dish was boiled for 1 min. on a small flame, 1 ml. of conc. nitric acid added with stirring and the solution boiled for a further 1 min. After cooling for 1 min. 0·5 ml. of 0·2 N-bromine water was added with stirring to decolorize the excess nitroso-R-salt, and the solution allowed to stand for 5 min. The excess bromine was removed by boiling for 1 min., and on cooling the volume was made up to 10 ml. with glass-distilled water. The colour was compared with similarly treated standards by using a Hilger spectrophotometer (blue-green filter); blank determinations, which showed no detectable cobalt content, were made up by treating 2 ml. of combustion mixture exactly as the samples to be analysed. Added cobalt was found to be recovered quantitatively from 1 to 10 μg./sample in the presence of a bacterial suspension of twice the strength of that encountered in the analyses, and at this concentration 15 μg. iron caused no deflexion from the standard curve.

RESULTS

Preliminary testing of the action of different cations

The basal partially-defined medium on which the G 12/6 strain produced a maximum yield of toxin, namely, one containing 0·20–0·24 μg. iron/ml. did not allow detectable toxin formation when a further 0·6 μg. iron/ml. (to give a concentration of $1{\cdot}25\times10^{-5}$M-$FeSO_4.7H_2O$) was added. Any significant effect of the salts of the other metals should, therefore, be detectable at a concentration of 5×10^{-5}M, which was used to test a variety of cations for their ability to inhibit the formation of toxin. In some instances the addition of this concentration of salt caused a decrease in the pH of the medium, in which case this was readjusted before autoclaving. Duplicate cultures were grown for each cation tested, and were harvested by centrifugation after 7 days of incubation at 34°.

In assessing the results shown in Table 1, the best estimate of growth, i.e. total cellular synthesis, is given by the sum bacterial-N+filtrate protein-N. This is a reasonable measure since there was no evidence that any appreciable proteolysis occurred in the cultures up to 7 days. Where the figures for bacterial-N were lower than those in the control cultures but the sum of bacterial-N+filtrate protein-N was similar to that in the control cultures, it was assumed that the added cation had accelerated lysis of the organisms. The validity of this assumption was confirmed by the fact that in such cases the toxin and coproporphyrin concentrations in the filtrate were usually comparable with those of the controls.

Table 1. *The effect of different cations on the growth, lysis, toxin and coproporphyrin of cultures of* Corynebacterium diphtheriae, *strain G* 12/6

G 12/6 strain of *C. diphtheriae* was grown for 7 days at 34° in 30 ml. of partially-defined medium. Salts were added to 5×10^{-5}M with adjustment when required, before autoclaving.

Cation added	Toxin titre (Lf/ml.)	Bact.-N (μg./ml.)	Filtrate Protein-N (μg./ml.)	Coproporphyrin (μg./ml.)
No addition	76	300	100	5·4
Cu^{++}	78	300	89	5·8
Ag^{++}	78	320	112	4·6
Zn^{++}	85	247	196	5·9
Cd^{++}	Growth only just detectable		.	.
Al^{++}	78	323	95	5·6
Pb^{++}	63	334	81	5·2
Cr^{++}	48	185	116	5·8
Mn^{++}	58	272	137	4·7
Co^{++}	34	272	108	4·6
Ni^{++}	80	320	99	5·3

The effects resulting from the addition of different cations show a marked variation. Cd^{++} was so toxic that only a barely detectable pellicle of organism appeared, and no analyses were made. Cr^{++} caused a moderate inhibition of growth and a decrease in toxin yield, though the coproporphyrin concentration in the culture filtrate was unaffected. Cu^{++}, Ag^{++}, Al^{++}, Ni^{++} all caused no significant deviation in growth, lysis or toxin titre from the controls, though Ag^{++} caused a decrease in the coproporphyrin concentration. Mn^{++} caused no growth inhibition as judged by total protein-N found, though lysis was accelerated, and the toxin and coproporphyrin concentrations were decreased. Co^{++} showed the most marked decrease in toxin yield, though growth was not inhibited appreciably. Zn^{++}, unlike any of the other cations, caused an overall increase in bacterial protein, reflected in increased toxin and coproporphyrin yields, and a marked increase in bacterial lysis. Pb^{++} caused no growth inhibition but showed a decrease in toxin and coproporphyrin yield.

Spectroscopic examination of the culture filtrates revealed an alkaline absorption spectrum (four-banded) of free porphyrin in every case except those cultures with added Zn in which there were typical two-banded metalloporphyrin spectra with peaks at 570 and 536 mμ. Thus in all other cases not more than a small fraction of the porphyrin was found in combination with the added cation, and the method of coproporphyrin analysis used would have resulted in the whole of the acid-labile zinc coproporphyrin complex being determined as free porphyrin. However, the possibility was not excluded that some of the small decreases of coproporphyrin found in the case of other metals which form acid-stable porphyrin complexes might have been due to a fraction of the coproporphyrin being held as unextractable metalloporphyrin. The results confirmed the observations of Mueller (1941) in respect of Mn^{++}, and also indicated that the effects of Co^{++}, Zn^{++} and Pb^{++} might merit further investigation. Zinc salts were found in a subsequent experiment to be active at much lower concentrations than those used here (1×10^{-5}M), but since their effect appeared to be solely one of accelerating lysis and they did not lead to any decrease in toxin titre, this effect was not followed up. The medium used in this experiment was deficient in Zn and Co when growth was in Pyrex flasks, since maximum titres of 90–100 Lf units/ml. were obtained by the addition of these two ions at 0·1 μg./ml. or by the use of soda-glass vessels for growth.

The effect of increasing concentrations of Co^{++}

Since the effect of Co^{++} on toxin formation appeared to be less than that of Fe^{++} at similar concentrations, the cobalt content of the organisms was investigated. It had been found previously that about half the Fe present in the medium at any concentration up to that necessary to inhibit toxin formation was to be found in the saline-washed organisms. The centrifuged organisms from a culture with added cobalt contained a large proportion of easily removable cobalt as demonstrated by the results shown in Table 2. Since the free culture medium contained in the plugs of centrifuged organism was

Table 2. *Removal of cobalt from* Corynebacterium diphtheriae *by washing with buffered* 1 % (*w*/*v*) NaCl *solution*

Organisms from 30 ml. of culture of *C. diphtheriae*, strain G 12/6, containing 10 μg. Co^{++}/ml. were washed with ice-cold 1 % (w/v) NaCl buffer (pH 7·0). After warming in a 45° water bath for 1 min. and 30 min. at room temperature the organisms were centrifuged down at 3000 rev./min. for 1 hr.; the volume of deposited organisms was 0·6–0·8 ml.

Treatment			
Unwashed	Washed × 1	Washed × 2	Washed × 3
Co^{++} organisms from 30 ml. culture (μg.)			
13·3	4·5	3·2	3·4
13·2	4·8	3·5	3·5
12·9	5·0	3·5	3·4

considerably less than their volume of 0·6–0·8 ml. (≡6–8 μg. Co^{++}), the 10 μg. Co^{++} which was removed by 3 washings must have contained some Co^{++} from the organisms themselves. This concentration of Co^{++} in the organisms is probably related to that found in yeasts (Nickerson & Zerahn, 1949).

Cobalt concentrations added, up to 6·4 μg./ml. medium were tested in duplicate cultures, being harvested after 7 days growth at 34°. The results in Table 3 show that, especially at the higher concentrations of Co^{++}, only a small proportion of the metal was in the organisms, and confirm that the decrease in toxin titre was disproportionately greater than the inhibition of growth and coproporphyrin formation. The basal medium, apparently, contained a suboptimal concentration of Co^{++}, since the addition of 0·05 μg./ml. stimulated growth and also toxin and coproporphyrin formation. When related to the concentration of metal in the organisms Co^{++} appears to have a greater effect on toxin formation than iron (see Clarke, 1958).

Table 3. *Effect of cobalt concentration in medium on cobalt content of organisms grown therein*

Cultures (30 ml.) of *Corynebacterium diphtheriae* strain G 12/6 were grown for 7 days at 34°. The organisms were centrifuged down and washed three times with 1% (w/v) NaCl buffered pH 7·0 before Co determination.

Co added (μg./ml.)	Toxin titre (Lf/ml.)	Bacterial-N (μg./ml.)	Filtrate protein-N (μg./ml.)	Bacterial Co (μg./ml.)	Coproporphyrin (μg./ml.)
—	74	360	71	0·005	5·2
0·05	86	393	78	0·027	5·5
0·4	68	347	60	0·047	4·5
1·6	39	295	52	0·080	4·3
3·2	14	—	—	0·107	3·3
6·4	10	233	81	0·083	2·2

The effect of cysteine and histidine on the activity of cobalt

Schade (1949) reported that cobalt at low concentrations inhibited the growth of many micro-organisms. He investigated its effect on the growth of *Proteus vulgaris* in particular, and found that the toxicity depended on the composition of the medium. Histidine reversed the inhibition competitively in the ratio of 2 molecules for every atom of Co present. This was interpreted as being due to the formation of a Co-histidine complex (Burk, Hearon, Caroline & Schade, 1946), which is formed at 37° and pH 7·5, and is stable under these conditions. Cobalt is also known to form complexes with cysteine (Michaelis, 1929; Schubert, 1931). It was considered that the presence of appreciable concentrations of histidine and cysteine in the medium used here might account for the fact that only a small proportion of the cobalt added to the medium was, apparently, taken up by the organisms during growth. The effect of the addition of each amino acid was therefore investigated (Table 4). It would appear that the amino acid-cobalt complexes are not stable under the conditions of growth, since the preformed complex with histidine was not less active than $CoCl_2$, and neither cysteine nor histidine caused any decrease in the activity of Co^{++}.

Table 4. *The effect of histidine and cysteine on the activity of cobalt*

Cultures of *Corynebacterium diphtheriae* strain G 12/6 (30 ml.) were grown for 7 days at 34°. The amino acids and cobalt were added before autoclaving, and the sterile histidine-cobalt complex after autoclaving of the medium.

$CoCl_2$ added (M × 10^5)	Histidine-HCl added (M × 10^5)	Co-histidine complex added (M × 10^5)	Cysteine-HCl added (M × 10^5)	Bacterial-N (μg./ml.)	Toxin (Lf/ml.)	pH value
—	—	—	—	400	87	7·62
5	—	—	—	360	35	7·66
5	10	—	—	357	35	7·75
—	10	—	—	417	87	7·20
—	—	5	—	333	35	7·39
80	—	—	—	162	5	7·51
80	160	—	—	159	3	6·81
—	160	—	—	420	88	7·48
—	—	80	—	187	3	6·52
—	—	—	—	360	74	7·38
—	—	—	160	363	78	7·26
10	—	—	—	233	10	6·72
10	—	—	160	236	10	7·26

The effect of vitamin B_{12}

Since cobalt is a component of the vitamin B_{12} molecule, 7-day cultures with and without added cobalt were analysed for this growth factor, the assays being carried out in Glaxo Laboratories by kind premission of Dr W. F. J. Cuthbertson. A culture grown on medium to which no cobalt had been added was found to contain 0·0097 μg. vitamin B_{12}/ml. (5% of it in the organisms), and one from medium containing added Co^{++} at 8 μg/ml. contained 0·15 μg. vitamin B_{12}/ml. (83% in the organisms). Since organisms grown in this concentration of cobalt contained 0·1 μg. cobalt/ml. and vitamin B_{12} contains 4% Co, only 6% of that cobalt was present in the vitamin B_{12} molecule. It was confirmed that vitamin B_{12} was not responsible for the decrease in toxin formation in medium to which cobalt was added, since, while having a slight growth-augmenting effect, addition of this vitamin had no effect on toxin formation at least in concentrations up to 1·6 μg./ml. (Table 5).

Table 5. *The effect of adding vitamin* B_{12} *on toxin formation by* Corynebacterium diphtheriae

Vitamin B_{12} was added as 'Cytamen 50' after sterilization to 30 ml. cultures of *C. diphtheriae* strain G 12/6, which were grown for 7 days at 34° and the organisms then harvested.

Culture	Bacterial-N (μg./ml.)	Toxin (Lf/ml.)
Control (i), no added vitamin B_{12}	407	84
Control (ii), no added vitamin B_{12}	410	87
0·05 μg. vitamin B_{12} added	443	84
0·20 μg. vitamin B_{12} added	423	85
0·80 μg. vitamin B_{12} added	407	90
1·60 μg. vitamin B_{12} added	410	89

The effect of cobalt on the iron concentration for maximum growth and toxin formation

Since it was found previously that only half the iron present in medium at any concentration up to 0·86 μg./ml. was found in the organisms, it was thought possible that the effect of adding cobalt might be that of combining with metal-complexing molecules in the medium, and thus allowing all the iron to remain freely available to the organisms. A series of cultures was therefore grown in which the effect of iron in the presence of increasing cobalt concentration was determined. If the action of cobalt was that of allowing the organisms to take up a higher percentage of the iron present in the medium, then the iron concentration required for maximum growth might decrease as the cobalt concentration was increased. The results in Table 6 show that the reverse occurred, allowing for the fact that in the presence of excess iron, cobalt itself was growth-limiting up to a concentration of 0·1 μg./ml. The iron concentration required for maximal toxin yield was unaffected by the addition of cobalt.

Table 6. *The effect of cobalt on the iron concentration of maximum growth and toxin formation by* Corynebacterium diphtheriae *strain G* 12/6

Cultures (30 ml.) were incubated for 7 days at 34°. $FeSO_4$ and $CoCl_2$ were added before autoclaving th medium.

	Cobalt concentrations							
	Nil		0·1 μg./ml.		0·4 μg./ml.		2 μg./ml.	
Iron added (μg./ml.)	Toxin (Lf/ml.)	Bacterial-N (μg./ml.)	Toxin (Lf/ml.)	Bacterial-N (μg./ml.)	Toxin (Lf/ml.)	Bacterial-N (μg./ml.)	Toxin (Lf/ml.)	Bacterial-N (μg./ml.)
Nil	57	260	57	235	47	207	23	186
0·03	63	327	65	333	56	305	22	293
0·06	80	413	80	447	63	377	38	338
0·09	80	413	85	480	69	463	46	367
0·12	77	457	77	480	69	480	46	386
0·15	68	467	69	480	55	487	38	391
0·20	53	450	53	463	46	473	26	420
0·30	28	437	25	443	22	457	14	357

The effect of cobalt on the cytochrome b and total iron content of diphtheria bacilli

Although cobalt does not act passively in providing more available iron in the medium it seemed possible that it might cause more of the iron of the organisms to be directed to the system responsible for inhibiting toxin formation; if Pappenheimer's suggestion were correct, this would be cytochrome *b*. It was therefore considered relevant to see whether adding cobalt caused any increase in cytochrome *b*, and also whether cobalt was present in the organisms as a haem complex which might have an action similar to haemin. Iron coproporphyrin III and protoporphyrin IX (haemin) complexes are capable of causing a decrease of toxin titre, though they are not as active as $FeSO_4$ in this respect (Clarke, 1958). The cobaltous complexes of coproporphyrin III and protoporphyrin IX (prepared by heating the free porphyrin in dilute NH_4OH solution with the theoretical quantity of $CoCl_2$) when similarly tested were found to be inactive up to a concentration of 1 μg. cobalt/ml., at which concentration $CoCl_2$ caused a 40 % decrease in toxin titre. The inactivity of the coproporphyrin complex, which is readily formed on autoclaving medium containing both components, was demonstrated by the fact that while cobalt and coproporphyrin both readily inhibited growth of *Corynebacterium diphtheriae* (coproporphyrin probably by combination with essential trace elements), added together in equimolar concentration they caused no growth inhibition.

When organisms grown in a medium containing 10 μg. cobalt/ml. were examined spectroscopically in the visible spectrum, in pyridine and reduced with sodium dithionite there appeared to be no qualitative or quantitative difference between the absorption bands of these organisms and those from control cultures, indicating that the addition of cobalt had not led to a conversion of more iron to haem enzymes, nor was there any appreciable cobalt porphyrin complex present. Washed organisms suspended in 0·85% (w/v) NaCl solution containing cobalt coproporphyrin III complex took up this complex readily, though the uptake was inhibited by adding culture medium.

To determine whether cobalt was present in the organisms as porphyrin complexes, a batch of organisms grown in a medium containing 10 μg. Co^{++}/ml. was washed three times with 1 % (w/v) NaCl solution buffered at pH 7·0 and extracted by the method of Rawlinson & Hale (1949). The flow-sheet (Table 7) shows the method used and distribution of cobalt between the fractions. There appeared to be no appreciable cobalt present as unbound porphyrin complex (ethanol soluble) or as bound complex removable from the

Table 7. *The extraction and fractionation of cobalt from* Corynebacterium diphtheriae *strain G* 12/6

- Saline-washed organisms (170 μg. Co)
  - Washed repeatedly with ethanol, acetone and 80 % (v/v) acetone in water; at 34°
    - *Extracts* (< 2 μg. Co)
    - *Residue* — Extracted repeatedly with acetone (1·0 vol.) + 0·2 vol. 1·5 N-HCl; at 34°
      - *Extract* (83 μg. Co) — Extracted repeatedly with ether + dil. HCl
        - *Ether extract* — Washed successively with 0·5 N-HCl and 3 % NaCl
          - *Washed extract* (< 2 μg. Co)
        - *Residual solution* (75 μg. Co)
      - *Residue* (92 μg. Co) — Washed at room temperature with 1 % (w/v) NaCl, buffered at pH 7·0
        - *Residue* (58 μg. Co)

organisms by HCl + acetone, nor was any visible by spectroscope as pyridine haemochromogen.

The lack of detectable increase in the absorption spectrum of organisms containing cobalt when viewed with a Hartridge reversion spectroscope indicated no increase in cytochrome *b* content, but this required confirmation. The cytochrome *b* content of these organisms and of control organisms, both from cultures containing only 0·2 μg. iron /ml., was not high enough to allow accurate determination to be made on ultrasonic disintegrated organisms as has been used for those grown in a higher iron concentration, but the extraction procedure with HCl + acetone could be used. Cultures were grown in rectangular soda-glass bottles, duplicate bottles containing 400 ml. of the basal partially-defined medium being harvested as follows:

(1) Optimum iron concentration (0·2 μg./ml.)—(*a*) with no added cobalt, (*b*) with 5 μg. Co^{++}/ml. (added as $CoCl_2$).

(2) Iron concentration of 0·8 μg./ml.—(*a*) with no added cobalt, (*b*) with 5 μg. Co^{++}/ml. (added as $CoCl_2$).

The organisms were harvested after 5 days of incubation at 34°, since it had been found previously that maximum growth was reached in this time and that longer incubation led to lysis, particularly of cultures containing higher iron and cobalt concentrations. Saline-washed organisms were analysed for cobalt, nitrogen, haemin, iron and cobalt, and coproporphyrin and toxin determinations were made on the culture supernatant fluids.

Table 8. *The effect of cobalt on cytochrome b content of* Corynebacterium diphtheriae *strain G* 12/6

The batches (2 × 400 ml.) of each different culture were harvested after 5 days of incubation at 34°. The haemin values given are those from extraction with HCl + acetone from defatted organisms. Values are in terms of units given/ml. culture.

Fe concn. in medium (μg.)	Co added (μg.)	Bacterial-N (μg.)	Bacterial-Fe (μg.)	Bacterial-haemin (μg.)	Bacterial-Co (μg.)	Toxin (Lf)	Copro-porphyrin (μg.)
0·2	Nil	476	0·135	0·044	—	76	3·6
0·2	5	371	0·185	0·037	0·39	22	2·7
0·8	Nil	555	—	0·46	—	11	0·90
0·8	5	550	—	0·31	0·23	2–3	1·70

The growth inhibition caused by cobalt in medium with a low iron concentration was prevented by the increase of iron concentration to 0·8 μg./ml. (Table 8). This concentration of cobalt, however, caused a decrease in the haemin content of the culture. Since the haemin content/unit bacterial-N of the low-iron cultures was unaffected by adding cobalt, it is possible that cobalt caused a decrease in cytochrome *b* synthesis which in an iron concentration of 0·2 μg./ml. was growth-limiting, but in an iron concentration of 0·8 μg/ml. was not.

From the results of the iron and cobalt analyses on these organisms it would seem that the presence of cobalt in the medium enhanced the total uptake of iron by the organisms though their haem content was not increased, whereas the higher the iron content of the medium the smaller the proportion of cobalt taken up. (The methods of analysis used for these two metals were insensitive to alterations in concentration of the other.) To understand whether the uptake of these two metals is in any way connected would involve a more detailed investigation. The decreases in toxin titre caused by 0·6 μg. iron/ml. and 5 μg. cobalt/ml. were not additive when both were present in the same culture. This was confirmed by a further experiment, where incubation for 7 days allowed maximum toxin production (Table 9). It is clear that the toxin decrease caused by either metal was dependent on the concentration of the other present in the medium, which is evidence of relationship between their modes of action.

Table 9. *The mutual dependence of the action of iron and cobalt on toxin formation and growth of* Corynebacterium diphtheriae *strain G* 12/6

The cultures were incubated for 7 days at 34°. Values are in terms of units given/ml. culture.

Fe concn. in medium (μg.)	Co added (μg.)	Toxin titre (Lf)	Bacterial-N (μg.)
0·2	Nil	95	577
0·2	2·0	37	498
0·4	Nil	16	480
0·4	2·0	11	540

DISCUSSION

In addition to iron, low concentrations of cobalt, lead or manganese depress the formation of toxin without materially affecting growth; cobalt and manganese also cause a slight decrease in coproporphyrin formation. The addition of 0·05 μg. cobalt/ml. causes an increase of growth and toxin and coproporphyrin formation. At higher concentrations of cobalt in the medium, growth is inhibited as well as toxin formation provided the iron content is low. When, however, the iron concentration is increased to 0·8 μg/ml., cobalt inhibition is prevented. Cobalt causes a decrease in haem content of the organisms at both iron concentrations, but, since the haem content is not the growth-limiting factor at the higher iron concentration, there is no growth inhibition. Preliminary observations of iron content of organisms in such cultures indicate that there is no overall decrease in iron content, but cobalt causes a change in metabolism resulting in a decrease in the amount of iron bound to the porphyrin nucleus.

There is further evidence that the effects of iron and cobalt are related in that the toxin decrease caused by either metal is dependent on the concentration of the other in the medium. Evidence has already been presented (Clarke, 1958) which indicates that the effect of iron in decreasing the formation of toxin by the G 12/6 strain of *Corynebacterium diphtheriae* is not associated quantitatively with the formation of cytochrome *b*. No serological relationship between diphtheria toxin and diphtherial cytochrome *b* has been obtained (Pappenheimer & Hendee, 1947). Neither has it been found possible to show any interference in cytochrome *b* activity in toxaemic muscle, though the effect of toxin in the silkworm appears to implicate the cytochrome system (Pappenheimer & Williams, 1952).

No assessment of the role of iron in the formation of diphtheria toxin is complete without consideration of the reports of a similar phenomenon in the case of other exotoxins though it may seem unlikely that these toxins are in any way similar in their activities. When *Clostridium tetani* was grown on a casein hydrolysate medium (Mueller & Miller, 1943) the concentration of iron had to be decreased to 0·03 μg./ml. for maximum toxin yield. Rogers & Knight (1946) found that in the presence of protein-free 'toxigenic factor' the maximum yield of α-toxin by *C. welchii* was obtained on a medium containing 0·3 μg./ml. of iron. The addition of 0·08 μg./ml. above this concentration resulted in a 50% decrease in toxin yield. No cytochrome system has been demonstrated in Clostridia. Van Heyningen investigated the neurotoxin of *Shigella shigae*, and found results which appear to be comparable with those found with *Corynebacterium diphtheriae*. When the organism is grown on a medium deficient in iron, the metal is almost quantitatively converted into haem; the addition of more iron leads to an increase of haem and neurotoxin formation. When, however, the iron concentration is increased further, the metal is taken up and is converted into a form in which it reacts with 8-hydroxyquinoline (i.e. is loosely bound in the cell) and the yield of neurotoxin falls (van Heyningen, 1955*a*). Moreover, this effect of iron, which is apparently not related to haem formation, is reproduced by cobalt and nickel. These two metals do not affect the respiration of the organisms but iron does (van Heyningen, 1955*b*).

Lascelles (1956) showed that cobalt inhibited the synthesis of iron protoporphyrin from α-aminolaevulic acid by suspensions of *Rhodopseudomonas spheroides*. This supports the view that the decrease of iron protoporphyrin in cultures of *Corynebacterium diphtheriae* containing added cobalt may be a direct result of the inhibition of haem synthesis. It seems a reasonable working hypothesis that this action of cobalt releases more of the total iron of the culture medium to be available for an iron system resonsible for the non-appearance of toxin in the culture filtrate. The total possible toxin inhibition by cobalt in any medium is governed by the total iron content, the degree of inhibition of haem synthesis and the proportion of the non-haem iron which is directed into this specific system.

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A Solution-Culture Technique for Obtaining Root-Hair, or Primary, Infection by *Plasmodiophora brassicae*

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SUMMARY: Primary infections were obtained by growing cabbage seedlings in a modified Hoagland's solution in which resting spores of *Plasmodiophora brassicae* Woron. were suspended. Seeds were germinated on filter paper wet with tap water, and after 2 days the plants were transferred to small glass tubes bent in the form of a shallow U or to small vials containing solution and spores. Zoosporangia were formed after several days growth at 25° in the dark. They were stained in aceto-carmine. A roughly linear relationship was found between the logarithm of number of infections/root and the logarithm of spore concentration in the medium. Numbers of infections were usually greater at 1/5 or 1/25 dilution of the culture solution than at the standard concentration, but were very much fewer or none in more dilute 1/125 or more concentrated (×5) solutions. The concentration which permitted maximal infection tended to vary from one experiment to another. Infection was not affected by changing from pH 5 to 6 but was greatly decreased at pH 8.

There is abundant evidence that *Plasmodiophora brassicae* Woron., the parasitic slime mould which causes the clubroot disease of crucifers, survives by means of the many spores it produces in the clubroot galls. When the gall decays, the spores are released into the soil where they may persist for many years. So far as is known, all new infections originate from these resting spores, so that factors which affect their infectivity are fundamental to any study of the parasitism of *Plasmodiophora brassicae.* Two kinds of test have been used in investigating the effects of environment on infection by *P. brassicae.* The older, and formerly the only, method is to enumerate or make observations on plants which develop clubroot symptoms when grown for several weeks in infested soil. The second method is based on the early stages of the life cycle of the parasite in roots of seedlings which have been grown for a few days in an infested medium. When a resting spore germinates in a situation favouring infection, the zoospore that escapes enters a root hair of the host plant and there develops into a group of zoosporangia. On a length of root, these groups or sori of zoosporangia can be stained and infections readily recognized and counted (Samuel & Garrett, 1945).

As there are two motile stages in the life cycle of *Plasmodiophora brassicae,* it will be convenient to call the zoospores which emerge from resting spores, primary zoospores and those from zoosporangia in the root hairs, secondary zoospores. Infection by a primary zoospore will be called primary infection or, as formerly, root-hair infection. The stages of the life cycle between the discharge of the zoosporangia and the formation of the plasmodia in the growing gall are unknown, but there is no evidence that infections by resting spores ever develop in any way other than to zoosporangia first. This is supported by the fact that the number of primary infections is affected qualitatively by soil conditions in much the same way as is the number of clubbed plants (Samuel & Garrett, 1945). Both methods seem to give a measure of infection from resting spores, but this is true only in so far as clubbing depends on primary infection. Factors, such as variation in host and pathogen, can influence or determine the development of clubs independently of the number of primary infections (Macfarlane, 1955). In this connexion we may recall that *P. brassicae*, although not known to complete its life cycle except on crucifers, can infect and form zoosporangia in various non-crucifers. Pryor (1940) showed that mineral nutrition of the host also affected the development of clubs, and there is some evidence that it can do so after the zoosporangial stage (Macfarlane, 1952). These considerations make it clear that each kind of test has a different range of application. For evaluating effects of environment on primary infection alone, the method of counting zoosporangial sori in root hairs is clearly the appropriate one. The present paper is concerned with the development of this technique. In earlier work primary infections were obtained by growing plants in soil, but for physiological studies this is too complex and simpler media are needed so that conditions can be more precisely defined. A method has therefore been evolved for infecting seedlings growing in nutrient solution. This paper describes the method, and some of the factors which affect the number of primary infections in these cultures.

METHODS

Host plant. Cabbage, horticultural variety 'King of Hearts' (Ryder & Sons Ltd., St Albans), was used in all experiments. Seeds were germinated on filter-paper, supported above and dipping into tap water in a 14 cm. Petri dish, and incubated in the dark at 25° for 48 or 60 hr. depending on the batch of seed. The radicles were then 5–10 mm. long and the seedlings could be fairly easily handled. Plants were selected by eye for uniformity; when size differences could not be eliminated in this way, seedlings of all grades were allocated to each experimental treatment.

Culture solution. The mineral nutrient solution for culture of seedlings was that described by Hoagland & Snyder (1934), but with the phosphate concentration doubled. The composition of the standard solution was thus (M): $Ca(NO_3)_2$, 0·005; KNO_3, 0·005; $MgSO_4$, 0·002; KH_2PO_4, 0·002. For convenience this solution will be referred to as S solution and other concentrations as 5S, S/5, S/10 and so on. The pH value of the S solution, measured with a glass electrode, was 5·0. The reaction of the solution was altered by supplying the standard amount of phosphate as appropriate proportions of KH_2PO_4 and K_2HPO_4. Doing this inevitably altered the potassium content, and when all the phosphate was given as the dibasic salt, it was changed to the maximum extent, viz., 1·3 times the standard amount. All culture solutions were made up with glass-distilled water, which had been equilibrated with the atmosphere with respect to dissolved gases by aeration overnight with air drawn from outside the building.

Inoculum. Resting spores of *Plasmodiophora brassicae* were obtained from clubroot galls grown in a warm greenhouse. Cabbage seedlings were planted when 2–3 days old in a mixture of 1 vol. loam soil to 3 vol. sand (pH of 5·5–6·0) in ordinary flower pots. Inoculum of crushed clubroot galls of spore suspension was poured on to the soil surface. The soil was kept wet and mineral nutrients were supplied in solution. Clubs were harvested about 6–8 weeks after inoculation. Most experiments were done with an isolate of *P. brassicae* which had been maintained on cabbage in the greenhouse for many years. In one experiment an isolate from Agdell field, Rothamsted, multiplied variously on cabbage and turnip, was used. These isolates are called I and II, respectively.

Maturation of the spores is apparently complete and extraction of spores easiest when the gall is beginning to decompose (see also Ayers, 1944). At this stage the cut surface of a gall usually has a grey or marbled appearance. After thorough washing and removal of fibrous roots, galls were finely ground in a mortar or macerated in a blender (MSE Atomix) with water. The brei was then filtered through muslin and, when necessary, the fluid was expressed by hand. The many spores which adhered to the muslin were discarded, but the pulp was resuspended in water and the extraction process repeated. The spores were washed in distilled water by centrifugation at least twice and usually five times. They were stored as dense suspensions in the refrigerator at 3–4°. Spores have been kept in this way for over 3 years now without serious loss of viability; Bremer, Wehnelt & Brandenburg (1937) also stored inoculum in this way.

The concentration of spores in suspensions was determined by haemocytometer counts made on appropriate dilutions. Spores in some suspensions tended to clump, making counting inaccurate or impossible, but they dispersed when suspended in weak alkali (e.g. 0·01 N-NaOH) instead of in water. Suspensions for use were made by diluting stock suspension in aerated water.

Technique for obtaining primary or root-hair infections. In attempting to improve the root-hair infection technique, the first modification tried was to replace the soil + sand medium (Macfarlane, 1952) by sand alone. Enough S/5 solution containing spores was added to acid-washed sand to bring its water content to 80 % saturation. In other respects the original method was followed. Many infections were obtained in this way, and sand cultures were employed successfully in some experiments, but the lower layers of the sand tended to become waterlogged, which restricted root growth. Pryor (1940), who used sand culture to get clubbed plants, avoided this difficulty by using an intermittent siphoning arrangement with a continuous drip nutrient supply. With an organism like *Plasmodiophora brassicae*, which has a free-swimming stage, solution culture seemed the simple and obvious method. In early experiments, however, when seedlings were supported on cork rafts floating on S/5 solution + spores in a beaker, infection failed. Seedlings were infected by growing them between glass slides and filter paper soaked in a suspension of spores in S/5 solution, but this method was not used much because the variable extent to which plants pushed the paper from the glass affected results. Woronin (1878) infected seedlings by placing them in spore suspension in watch glasses;

this was repeated with S/5 solution, and numerous, though somewhat sporadic, infections were obtained. As proximity to the spores, which quickly settle to the bottom, and possibly a small volume, may have favourably influenced infection in the watch glass as compared with the beaker, small glass tubes, 75 mm. × 5–6 mm. internal diameter, bent in a shallow U-shape and open at both ends, were made for single plants. They were supported horizontally in racks cut from aluminium sheet. Vials, with dimensions 50 mm. × 8·5 mm. diameter, were also used. In the shallow U-tubes, seedlings were held in position by loose cotton wool plugs; in the vials they were supported on fine mesh stainless-steel gauze in which holes large enough for the roots had been punched with a stout needle. Abundant infections were obtained in these vessels. Seedlings in the infection tubes were kept in a moist chamber in the dark at 24–25°. Early stages of infection have been seen 36 hr. after setting up and zoosporangia were usually formed by the third or fourth day. After incubation for 4–6 days, roots were removed to aceto-carmine (Samuel & Garrett, 1945), which was made by adding 1 g. carmine to 100 ml. boiling 45% (v/v) acetic acid in water, filtering and diluting to 300 ml. with water.

Evaluation of results

Counting infections. Roots were mounted in water on a slide ruled at intervals to aid counting and examined microscopically with a 16 mm. objective and × 10 ocular. Constant change of focus was necessary to see all infections in root hairs lying on either side of the root. All infections seen were counted, including those in rhizodermal cells other than root hairs, either on a selected part of the root or, more usually, along the whole length. Counting infections along the whole root has the advantage of avoiding variations in susceptibility in different parts of the root (i.e. at different times during the growth of the root) which may be caused by experimental treatments. On the other hand, if root growth were arrested at an early stage, there might be fewer infections on the root simply because of a lack of cells. On balance, however, it seems simpler to regard the whole root as the unit which, as it were, traps parasites and to leave the question of the effectiveness of the trap for later analysis. Each group of zoosporangia was regarded as the consequence of a single infection, and where two or more distinct groups were seen in one cell they were counted as separate infections. No observations were made on the plasmodia from which zoosporangia developed to see whether they fused or not, but as apparent multiple infections were more numerous when there were very many infections, the separate sori probably indicate separate infections. This supports the view of Ayers (1944) who, from observations on infected root-hairs, concluded that plasmodia in the hairs did not fuse.

Statistical analysis. The standard errors of number of infections/plant for separate groups of observations were proportional to the group means. Before subjecting results to analysis of variance they were therefore transformed to a logarithmic function. The transformation log $(x+c)$, where $x=$number of infections and c is a constant, was tried on the results of several experiments and the scatter diagrams of standard errors plotted against group means were inspected. With $c=1$ the transformation tended to over-correct and a more satisfactory value of c seemed to lie somewhere between 10 and 25. Standard errors of groups with low means tended to be unduly low however, and, following Kleczkowski (1955), the transformation

$$z=\log_{10}\tfrac{1}{2}\,[x+c+\sqrt{(x^2+2cx)}]$$

was substituted. When $c=20$, this transformation satisfactorily eliminated error dependence on the mean. Values of z were obtained directly from Kleczkowski's table. For some experiments, values of c other than 20 were better, and for one standard errors were independent of the mean when $c=1$. The practical alternative to determining the best value of c for the results of every experiment is to select a value that seems to be generally suitable. Thus, in analysing the experiments described here, the value 20 was chosen. The graphs are based on actual numbers of infections and in one table both actual numbers of infections and transformed values are given. The term 'least significant difference' is sometimes abbreviated to 'L.S.D.'

RESULTS

Effect of spore concentration

Previously, when plants were infected in soil, a linear relationship was found between the logarithm of the mean number of infections on a segment of root and the logarithm of the spore concentration in the inoculum. A roughly similar relationship has now been found for plants infected in solution cultures. The results of several experiments are shown in Figs. 1 and 2 with both numbers of infections and spore concentration plotted on logarithmic scales. The points in Figs. 1 and 2A indicate numbers of infections/2nd cm. of root and/whole root, respectively, of plants infected in S/5 solution; those in Fig. 2B indicate infection on whole roots infected in S solution. The similar trends of the two groups of curves in Figs. 1 and 2A, which are based on different experiments, suggest that comparable results might be obtained by counting infections on either 1 cm. of root or on the whole root. When plants were infected in S/5 solution convenient numbers of infections for counting, say 50–300 infections/plant, were obtained by spore concentrations of $2\times10^5-2\times10^6$/ml. when only 1 cm. was examined and by $5\times10^2-5\times10^3$ spores/ml. when the whole root was examined. The number of infections obtained with a given concentration of spores, however, varied considerably from one spore suspension to another and from one experiment to another. Similarly, although when plants were in S solution higher spore concentrations were usually necessary to obtain the same number of infections that were obtained in S/5 solution, this effect was not invariable.

Effect of concentration of the culture solution

The results summarized in Fig. 3 and Table 1 show that this factor is important. Relative numbers of infections at different concentrations are shown in Fig. 3 as means over all experiments. In general, infection was suppressed at high concentrations of the culture solution and slightly less so at very low concentrations, with a high peak in numbers of infections at an intermediate level towards the dilute end of the scale. Table 1 shows that the response to changes in the concentration of the solution varied from one experiment to another. Infections were consistently fewer in the most dilute and in the most concentrated, than in other solutions. In S solution there were fewer infections than in S/5 solution in six experiments, in one there was no difference and in another there were actually more than in the dilute solution. Compared with

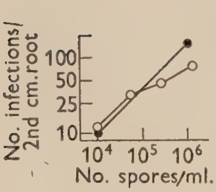

Fig. 1

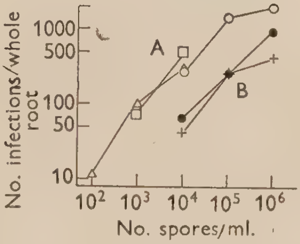

Fig. 2

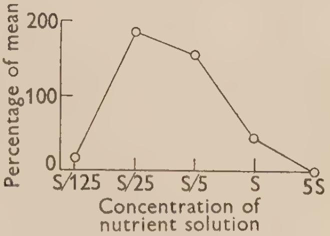

Fig. 3

Figs. 1 and 2. Effect of spore concentration on numbers of infections. Fig. 1. Cabbage seedlings infected in S/5 solution, pH 6. Two experiments: ○ with 8 replicates and ● with 5 replicates. Fig. 2. (A), cabbage seedlings in S/5 solution, pH 6; (B), in S solution, pH 6. Four experiments: ● ○ with 5 replicates. These points show the means of actual nos. infections in Expt. 8, Table 1. Means of transformed nos. infections are given in the table. □, with 12 replicates; △, with 10 replicates; +, with 12 replicates.

Fig. 3. Effect of concentration of the culture solution on numbers of infections. For each experiment, the no. infections/plant at any one concentration of the culture solution and at any one spore concentration, was expressed as a percentage of the mean for that spore concentration. The graph shows means of these percentages over all experiments.

Table 1. *Effect of concentration of the culture solution on numbers of infections*

Except in Expt. 5, cabbage seedlings were infected in solution cultures and infections were counted along the whole root. In Expt. 5 plants were infected in sand cultures with six replicate pots/treatment and 5 plants/pot and infections were counted on the 1st and 2nd cm. of root. Dash = not tested.

		Means of transformed nos. infections/plant. Concentration of solution					Least significant difference	No. of
Expt.	Spores (no./ml.)	S/125	S/25	S/5	S	5S	($P = 0{\cdot}05$)	replicates
1	$2{\cdot}5 \times 10^5$	—	2·65	2·61	2·14	1·03	0·22	12
2	10^5	—	1·85	2·95	1·59	1·07	0·40	4
3	5×10^3	—	2·26	1·85	1·11	1·00	0·30	10
4	5×10^3	1·07	1·46	1·81	1·11	1·00	0·20	20
5	4×10^5	1·46	1·70	1·93	—	—	0·20	6
6	5×10^4	—	—	2·47	2·69	—	0·49	5
7	10^4	—	—	1·83	2·42	—	0·34	10
8	10^4	—	—	2·46	1·84	—	0·31	5
	10^5	—	—	3·11	2·37	—		
	10^6	—	—	3·25	2·95	—		
9	5×10^3	1·04	1·77	1·27	1·12	1·00	0·24	7
	$2{\cdot}5 \times 10^4$	1·30	2·16	2·28	1·04	1·00		

S/25 solution there were also usually fewer infections in S except once when there was no difference. In the six experiments in which infection at concentrations S/5 and S/25 was compared, there was no difference in one; infection was greater in S/5 solution in three experiments and greater in S/25 in two. Within an experiment, variation of spore load sometimes influenced the effect of concentration of the solution. Thus in Table 1, Expt. 8, the difference in numbers of infections in S compared with S/5 solution was smallest at the highest spore concentration and was just no longer significant at the 5% probability level. As there were many infections at this spore load, it might be argued that the available infection sites under those particular conditions were becoming saturated. Something similar seems to have happened in Expt. 9, but at a lower level of infection and at different concentrations of the solution. Thus at the lower spore concentration there were far more infections in S/25 solution than in any other, but with more spores the number of infections in S/25 solution was equalled by that in S/5 solution. Under the conditions of this experiment, however, infection in S solution was not affected by an increase in spore concentration.

Some plants grown in the most dilute solution S/125 had dead cells over much of the root and dead root tips with laterals growing out behind, symptoms which strongly indicate calcium deficiency. Most plants had produced roots several cm. long before the tips died. Similar effects were occasionally seen on roots grown in S/25 solution. Roots grown in S/5, S and 5S solutions showed no obvious differences from one another.

In some solutions, changing the concentration also changed the pH value and in some experiments there was a gradient of pH values corresponding to a gradient in numbers of infections. The regression of numbers of infections on initial pH value determined on data from four experiments was, however, not significant ($P=0{\cdot}09$). Except for one 5S solution at pH 4·6, all pH values lay between 5·3 and 6·4. As shown in the next section, it seems unlikely that differences in pH of this magnitude influence infection.

Effect of pH *value*

Numbers of infections at three pH values, 5, 6 and 7, by two different isolates of *Plasmodiophora brassicae* are shown in Table 2. Isolate I was more infective than isolate II, but both were affected similarly by changes in pH value. At pH 7 infection was depressed relative to that in the other solutions, confirming the general view that infection by *P. brassicae* is favoured by acid conditions. Infections did not differ at pH 5 and 6.

Study of the effects of pH value itself on primary infection is complicated by other changes in the medium inevitably occasioned by alteration of acidity. The most important of these is probably the precipitation of calcium phosphate in the neutral and alkaline region, thus changing the concentration of both calcium and phosphate. Also, spores might be imprisoned in such precipitates. Other difficulties are the changes in pH value caused by the growing plants and the limitations on buffering imposed by the impossibility of growing plants in solutions of very high salt content. In an experiment in which infection at three concentrations of both acid and alkaline potassium phosphate was compared, an attempt was made to avoid large precipitates of calcium phosphate(s) by having in addition to one with the usual concentration a series with a very low concentration of calcium. Phosphate concentrations were increased above the standard concentration, 0·002 M, (S) to increase buffering capacity. Results and pH measurements are given in Table 3, which also indicates those alkaline solutions in which visible precipitates developed. There was no consistent effect of calcium on infection so the values for these treatments have been pooled. Measurements of pH value showed that, in the short duration of this experiment (4 days), buffering was reasonably adequate. The greatest change recorded was 1·3 units in the low acid-phosphate + high calcium solution. At the high concentration of the alkaline phosphate the seedling roots died; they grew at this concentration of the acid phosphate, but there was no infection. Results were therefore obtained from the medium and low (standard) phosphate treatments. Infection was depressed both by high pH values and concentration of phosphate above the standard. Thus, phosphate concentration had an effect similar to that caused by changes in the concentration of the rest of the solution, and the effect of pH value was again as expected. The effect of pH value was significantly greater at the medium than at the low phosphate concentration. The question then arises whether this result is actually due to differences in pH value. The removal of spores in calcium phosphate precipitates was probably unimportant. If removal of spores by sedimentation were a complicating factor, the results were the opposite of what would be expected, because spores settle more quickly in acid than in alkaline solutions. Differences in potassium content were small in comparison with changes in pH value. On the other hand, factors like CO_2 content, known to depend on pH, were not taken into account; nor were

Table 2. *Effect of* pH *value of culture solution on root-hair infection of cabbage seedlings by two isolates of* Plasmodiophora brassicae

Plants infected in S solution containing 5×10^4 spores/ml., in shallow U-tubes. Five replicates. pH values at end of experiment determined on bulked solutions from the five replicate tubes. L.S.D. = least significant difference.

	Means of transformed nos. infections/plant			
Initial pH value	Isolate I	Isolate II	Means	
5·0	2·60	1·89	2·24	L.S.D. (P=0·05)=0·41
6·0	2·65	2·29	2·47	
7·0	1·85	1·29	1·57	
	L.S.D. within table (P=0·05)=0·58			
Means	2·37	1·82		
	L.S.D. (P=0·05)=0·34			

Initial pH value	pH value at end of experiment	
5·0	6·0	6·0
6·0	6·5	6·5
7·0	6·9	6·9

changes in calcium known. Even at this lower concentration of calcium, the formation of nuclei of calcium phosphate at pH 8 would be virtually certain (Bjerrum, 1949), so that the concentration of free calcium ion might be considerably changed, although no precipitate was seen. The role of calcium in solutions of different pH value thus remains problematical.

Table 3. *Effect of* pH *value and potassium phosphate concentration on numbers of infections*

Cabbage seedlings infected in solution cultures. The basal solution contained 0·005 M-KNO_3 and 0·002 M-$MgSO_4$. Either KH_2PO_4 or K_2HPO_4 was added to 0·002 M, 0·0067 M or 0·02 M and $CaCl_2$ to 0·0005 M or 0·005 M. The low phosphate + high calcium solution thus corresponded to S solution with $CaCl_2$ instead of $Ca(NO_3)_2$. Spore concentration 10^6/ml. Four replicate U-tubes. Values for the calcium treatments have been pooled in the tables of nos. infections so that each figure is based on eight replicates. pH values at the end of the experiment were determined on bulked solutions from the four replicate tubes.

Mean no. infections/plant

Phosphate concentration (M)	KH_2PO_4	K_2HPO_4
0·0200	0	Roots died
0·0067	503	9
0·0020	2750	537

Means of transformed nos. infections/plant

Phosphate concentration (M)	KH_2PO_4	K_2HPO_4
0·0067	2·66	1·30
0·0020	3·36	2·61

Least significant difference ($P = 0·05$) = 0·31

KH_2PO_4 (M)	$CaCl_2$	pH values: Initial pH	pH values: Final pH	Visible precipitation of calcium phosphates
0·0200	High	4·85	5·35	None
	Low	4·80	5·35	
0·0067	High	5·00	5·55	
	Low	5·00	5·85	
0·0020	High	5·00	6·30	
	Low	5·05	5·85	
K_2HPO_4 (M)				
0·0200	High	8·10	8·25	Precipitate
	Low	8·60	8·20	None
0·0067	High	7·40	7·70	Precipitate
	Low	8·00	7·90	Slight precipitate
0·0020	High	7·20	7·00	Precipitate
	Low	7·40	7·30	None

DISCUSSION

The technique described for obtaining root-hair infections by *Plasmodiophora brassicae* in solution cultures has a number of advantages over the soil method described by Samuel & Garrett (1945). Solutions can be standardized and are more easily handled than soils, and the slow process of washing out roots from soil is avoided. Uniformly shaped vials are readily obtainable and the shallow U-tubes can be made at least fairly uniform. Each tube with its seedling is the unit of replication and as tubes or vials in their racks occupy relatively little space large experiments can be easily set up with as many replications as are necessary to give the precision desired. The principal drawback to all quantitative studies of primary infection is the labour of making the microscopical counts. It is therefore important to arrange that the mean number of infections is in a convenient range. Unfortunately, the considerable variation in numbers of infections which often occurs on different occasions, even with the same preparation of spores, makes the achievement of this condition uncertain. If the technique is to be fully exploited, this difficulty must be overcome. Some modification of the method of raising seedlings now seems likely to lead to improvements in this respect. At present it has been found convenient to use the S/5 solution for most experiments but if the method of preparing the seedlings were changed, another concentration of the culture solution might prove to be better. The method is obviously convenient for studying the early stages of the parasite in the root and the external factors which influence infection. Apart from the advantages already claimed and the simplicity of the infection medium there is probably little to choose between this method and that of Samuel & Garrett for such purposes as measuring the infectivity of variously treated spores and the preliminary testing of possible fungicides against *P. brassicae*. It also suggests ways of handling the organism which might be useful for life-cycle studies. For example, experience in infecting plants from resting spores *in vitro* should be valuable when investigating the behaviour of secondary zoospores. Finally, a technique such as this should help to achieve and use spores free from the contaminating micro-organisms which are such a serious nuisance in many kinds of work.

The results obtained when the concentration of the culture solution was varied have done little more than define the effect of this factor on infection. Obvious questions, e.g. whether one or more ion species in particular are always concerned or whether different ions are involved at different parts of the concentration range, whether the effect is on host or on parasite and what causes the variation between different experiments, remain unanswered. It may therefore be useful to discuss some of the lines of approach suggested by the above and other work. The signs of calcium deficiency in the roots grown in the very dilute solutions suggest that the decline in numbers of infections in this concentration range may be due to some effect on the host cells in which calcium is involved. The particular variations that were made in calcium content of solutions did not appear to have much effect on infection. In the sand-culture experiment (Table 1, Expt. 5) there were three solutions with different ratios of calcium to other ions, calcium being replaced by sodium. Each of these solutions was then tested at the three dilutions shown. There was a suggestion, though little more, that infections were fewer when calcium concentrations were low. Over the concentration range S/25 to S there was even less evidence about the effect of calcium. Changing the calcium concentration to one-tenth of the standard concentration had no consistent effect on infection (Table 3). At concentrations greater than the standard, the total salt concentrations attained and the effects caused by various treatments, together with some data from the experiments of Samuel & Garrett (1945), are shown in Table 4.

That making the concentration of the culture solution 5S or adding spores to soil in 0·1N-sodium or potassium chlorides suppressed infection suggests an effect not associated with a particular ion and hence perhaps due to the osmotic pressure of the solution. It is conjectured that *Plasmodiophora brassicae* might perhaps be affected by this. Potassium phosphate (Table 4) depressed infection with only a small increase in salt concentration. Perhaps potassium phosphate was more inhibitory than other salts or perhaps the response of infection to increasing salt concentration was merely beginning to be more pronounced at this concentration. The effect of increasing salt concentrations from S/5 upwards is strikingly similar to that obtained by Smith & Walker (1941) with *Aphanomyces euteiches* on peas. They ascribed the whole phenomenon to effects of salt nutrition on the pea host.

Table 4. *Effect of salt concentration on numbers of infections obtained in solution culture and soil culture*

Based on: (1) experiments quoted in Tables 1 and 3, in which plants were infected in solution culture; (2) experiments of Samuel & Garrett (1945) who added salts to spore suspensions which were then mixed with soil. Brassica seedlings were grown in the inoculated soils to obtain primary infections.

	Basal medium	Salt(s) added	Final total salt concentration in culture solution or spore suspension (g./l.)	Effect on nos. of infections relative to respective standard
Present experiments	S solution	None	1·837	Standard
		All basal (5S)	9·185	Decrease
		KH_2PO_4	2·612	Decrease
Samuel & Garrett (1945)	Water + spores (added to soil)	None	0·000	Standard
		0·01 N-NaCl	0·585	None
		0·01 N-KCl	0·745	None
		0·01 N-$CaCl_2$	0·555	None
		0·10 N-NaCl	5·850	Decrease
		0·10 N-KCl	7·450	Decrease

Recently, Palm & McNew (1956), from results of experiments in which crucifer seedlings were infected with *Plasmodiophora brassicae* in sand culture, reported that the number of primary infections was markedly affected by the calcium content of the nutrient solutions. Numbers of infections usually reached a maximum when the calcium content was 2 mg.equiv./l. (also the concentration of calcium in S/5 solution) and fell off rather steeply on either side of the peak. They also stated, without elaboration, that the calcium concentration at which infection reached a peak, depended on the concentrations of other ions. The similarity of these results to those obtained in the present experiments by changing the concentration of the whole solution is clear. In the present experiments, however, the concentration which permitted maximum infection differed on different occasions. This variation, and the fact that maximum infection could be at such widely different concentrations as S/25 and S makes one suspect that there is one underlying mechanism, at least until the highest concentrations are reached. Some factor at present uncontrolled must be involved and it seems possible that it may operate during the pretreatment of the seedlings. Seeds were germinated in tap water which in Harpenden contains, among other things, large amounts of calcium bicarbonate. It is possible that the assumption underlying the use of tap water for this purpose, namely, that variations in its content of dissolved material, whether calcium salts or other substances, were unimportant, may prove to be erroneous. These problems are being investigated. The possibility that calcium may be a factor in infection by *P. brassicae* is of especial interest in view of the participation of this element in the structure of all plant cell-walls and its important, if somewhat complex, effects on the growth of roots.

As it is virtually impossible to alter the pH value of the culture solution without varying other quantities some uncertainty must remain about the effect of pH on infection. There can be little doubt that it was the change of pH of the culture solution from 5 to 8 and not alterations of potassium content which depressed infection. This is in agreement with the demonstration by Samuel & Garrett (1945) that the inhibitory effect of lime and other alkalis on infection by *Plasmodiophora brassicae* in the soil is due, directly (or indirectly in the case of carbonates) to the addition of hydroxyl ions and not of the cations. In spite of this, Palm & NcNew (1956), on the basis of their sand-culture experiments, again raised the old and much debated question as to whether the effect of lime in controlling clubroot is in any way due to the calcium added. I do not think that it has yet been shown that adding calcium has an effect at all comparable to that which is obtained by adding alkalis and is generally believed to be caused by high pH values. But in any of the media that have been used for infection tests, changing the pH value even from slightly acid to slightly alkaline will also change the amount of free calcium ion. Thus, in nutrient solutions containing phosphates, the precipitation of calcium phosphate(s) in the alkaline region makes it difficult if not impossible to disentangle changes in pH from changes in free calcium content. In soils the situation must be more complex. These changes might or might not have effects on the plant and on infection but they do make it impossible to control the experimental conditions.

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The Influence of Chemical Structure on β-oxidation by Soil Nocardias

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SUMMARY: Studies of the β-oxidation of the fatty acid side-chain of certain ω-aryl- and ω-aryloxy-n-alkylcarboxylic acids by *Nocardia opaca* (strain T_{16}) and *Nocardia* sp. (strain P_2) show that γ-phenylbutyric is more rapidly oxidized than γ-phenoxybutyric acid. The effect of the oxygen bridge is even more striking when γ-(1-naphthyl)- and γ-(1-naphthyloxy)-butyric acids are compared. The rate of β-oxidation (by strain T_{16}) of 3-, 4-, and 2-isomers decreases in that order. This applies to monochloro- and monomethylphenoxybutyric acids and to monochlorophenoxypropionic acids. Substitution in position 2 has by far the greatest effect and chlorine exerts a bigger influence than a methyl group in all positions. The conversion of γ-(2-methyl-4-chlorophenoxy)-butyric acid (MCPB) and γ-(2:4-dichlorophenoxy)-butyric acid (2:4-DB) is very slow and proceeds only to β-hydroxy acids. On the other hand, ϵ-(2-methyl-4-chlorophenoxy)-, ϵ-(2:4-dichlorophenoxy)- and ϵ-(2-chlorophenoxy)-caproic acids are relatively rapidly converted to their corresponding butyric acid derivatives. With strain T_{16} ω-(2-naphthyloxy)-butyric and propionic acids are more rapidly converted to their corresponding acetic acids and phenols respectively than the ω-(1-naphthyloxy) compounds. The rate of β-oxidation of γ-phenyl-, γ-(3-indolyl)- and γ-(1-naphthyl)- butyric acids (by strain P_2) decreases in that order. It has been shown that β-hydroxy acid intermediates are formed from ω-aryloxybutyric acids and those from MCPB, γ-(2-methyl-4-chlorophenoxy)-crotonic and γ-(2-naphthyloxy)-butyric acids have been isolated and identified.

It was found (Webley, Duff & Farmer, 1957) that β-hydroxy acid intermediates accumulated during the conversion of 3- and 4-chlorophenoxybutyric acids to their corresponding acetate derivatives by washed suspensions of *Nocardia opaca*. Similarly, it was found (Webley, Duff & Farmer, 1955, 1956) that the breakdown of the side-chain of β-phenylpropionic acid and of α-(1-naphthyl)-hendecane gave $\alpha\beta$-unsaturated acids as intermediates. The coenzyme A derivatives (Fig. 1) of β-hydroxy and $\alpha\beta$-unsaturated acids are known intermediates in β-oxidation (Lynen, 1953; Mahler, 1953), but the free acids had not been previously detected. It seemed therefore that their production during β-oxidation by *N. opaca* of the compounds mentioned above was associated with ω-aryl and ω-aryloxy substitution. In the present work with *N. opaca* and an unidentified soil nocardia it has been found that the ring structure, the oxygen bridge and the position and nature of the ring substitutents affect the rate of β-oxidation and the accumulation of intermediates when the side-chain of the metabolite is short. Other β-hydroxy acids now identified include those from γ-(2-methyl-4-chlorophenoxy)-butyric acid (MCPB) and γ-(2-naphthyloxy)-butyric acid.

$$\begin{array}{l}
\mathrm{R.(CH_2)_{\mathit{n}}.COOH + CoA.SH + ATP} \\
\qquad\qquad\quad \downarrow \\
\mathrm{R.(CH_2)_{\mathit{n}}.CO.S.CoA + AMP + P\text{-}P} \\
\qquad\qquad\quad \downarrow \\
\mathrm{R.(CH_2)_{\mathit{n}-2}.CH{=}CH.CO.S.CoA + 2H} \\
\qquad\qquad\quad \downarrow \mathrm{+H_2O} \\
\mathrm{R.(CH_2)_{\mathit{n}-2}.CHOH.CH_2.CO.S.CoA} \rightarrow \mathrm{[R.(CH_2)_{\mathit{n}-2}.CHOH.CH_2.COOH]} \\
\qquad\qquad\quad \downarrow \\
\mathrm{R.(CH_2)_{\mathit{n}-2}CO.CH_2.CO.S.CoA + 2H} \\
\qquad\qquad\quad \downarrow \mathrm{+CoA.SH} \\
\mathrm{R.(CH_2)_{\mathit{n}-2}.CO.S.CoA + CH_3.CO.S.CoA} \\
\qquad\qquad\quad \downarrow \\
\mathrm{[R.(CH_2)_{\mathit{n}-2}.COOH]}
\end{array}$$

Fig. 1. Stages in the β-oxidation of substituted fatty acids. ATP = Adenosine triphosphate; AMP = adenosine monophosphate; P-P = pyrophosphate; CoA.SH = coenzyme A (reduced). In the present work $n=5$, 3 or 2 to give the corresponding substituted caproic, butyric or propionic acids and R = various aryl or aryloxy groups. Products isolated are indicated thus [ ].

METHODS

Organisms. Nocardia opaca strain T_{16} (Webley, 1954) and *Nocardia* sp. strain P_2 isolated from soil by Dr Treccani (University of Milan) were used throughout.

Production of organisms. Strain T_{16} was grown as described previously (Webley *et al.* 1955). In some experiments 1 % (w/v) phenylacetic acid (sodium salt) was used in place of glucose, 0·1 % (w/v) yeast extract (Difco) was added and the cultures were incubated for 7–12 days at 25°. Strain P_2 was grown similarly, but 1 % (w/v) glucose nutrient broth was used and the flasks were incubated for 14 days. The organisms were harvested with aseptic precautions for the large-scale incubation experiments.

Manometric experiments. Measurements of O_2 uptake were carried out as already described (Webley *et al.* 1955), substrates being tipped in from the side arm of the Warburg flasks after the overnight incubation required to decrease endogenous activity.

Large-scale experiments. These were carried out as in previous work (Webley, *et. al.* 1955). The flasks contained 25–100 ml. of 0·0133 M-phosphate buffer (pH 7·2), 0·004 M (unless otherwise stated) ω-aryl- or ω-aryloxy-alkylcarboxylic acid (sodium salt) and the equivalent of 200–800 mg. dry weight of washed glucose-grown organisms. The volume was usually determined by the amount of the substrate available. Organisms were added aseptically to the sterile substrate buffer mixture. When comparing the rate of conversion of different compounds equal samples of the same suspension were added to each flask. It was necessary in certain experiments to add fresh organisms at intervals of 7 days to bring about sufficient conversion of the substrates for chromatographic and spectrochemical analysis. These experiments were terminated 7 days after the final addition of fresh organisms. Since this type of experiment was often continued for several weeks strict aseptic technique was necessary. The contents of all flasks were examined microscopically and by streaking on nutrient agar plates after the removal of samples, at the end of the experiments, and before addition of fresh organisms in the longer incubation experiments. Any flask which became contaminated was discarded. Samples (5–25 ml.) or the whole contents of the flasks were taken when required and after removal of the organisms the supernatant fluids were acidified and extracted with ether. The ether-soluble products were subjected to chromatographic and spectrochemical analysis as described below. Sometimes the supernatant fluids were examined directly.

Spectrochemical techniques. Where the product was a single substance, it was identified from its infrared spectrum in the solid state, such spectra being highly specific, sensitive even to crystalline form. Mixed products were examined in dioxan solution 5 % (w/v) in 0·1 ml. optical cells since in this solvent interaction between the components of a mixture is avoided, as each component associates preferentially with solvent molecules. Under these conditions the β-hydroxy acid intermediates, the substituted acrylic acids, and the aryloxyacetic acids are each distinguished from other components by specific absorption bands. The alcoholic hydroxyl group of the β-hydroxy acid intermediates absorbs at 3420 cm.$^{-1}$ in dioxan solution, well separated from the absorption of acidic hydroxyl groups at 3070 cm.$^{-1}$, as shown in Fig. 2, for β-hydroxy-γ-(2-methyl-4-chlorophenoxy)-butyric acid. Estimates of concentration of β-hydroxy acid in mixtures were made by comparison with standards, the limit of detectability being about 10 % of the total acids.

Similarly, the infrared absorption band due to the C=C bond at 1666 cm.$^{-1}$ distinguished the acrylic acid intermediates from the other components (Fig. 3). About 5 % of γ-(2-methyl-4-chlorophenoxy)-crotonic acid was just detectable with certainty in mixtures with γ-(2-methyl-4-chlorophenoxy)-butyric acid, and a similar sensitivity is to be expected for other phenoxycrotonic acids. The carbonyl group of the aryloxyacetic acids (seven examples were examined) showed doublet absorption at 1770 and 1745 cm.$^{-1}$ in dioxan solution of which the higher frequency component is not given by either the aryloxybutyric or the β-hydroxybutyric acids (Fig. 3). This band permitted the estimation of phenoxyacetic acids down to 10 % in mixtures. The higher frequency component probably originates from a rotational isomer of the phenoxyacetic acid complex with dioxan, in which the oxygen of the carbonyl group is closest to the ether oxygen of the phenoxy group, whilst the lower frequency component corresponds to other rotational isomers in which interaction between the two oxygen atoms is slight. Similar effects have been noted when a halogen atom is in the α-position to a carbonyl group (Bellamy, Thomas & Williams, 1956), the carbonyl frequency being abnormally high only when the halogen is close in space to the oxygen atom.

Ultraviolet absorption spectrometry was used to follow the conversion of ω-aryloxypropionic acids to the corresponding phenols, by measurements on the supernatants of samples withdrawn from large-scale experiments. The total concentration of the aryloxy group was estimated by measurements in acid solution, in which the absorption of the phenols closely resembles the parent aryloxypropionic acids. In alkaline solution, the phenols give the intense distinctive absorption of their anions, whilst the spectra of the aryloxypropionates show negligible changes. Accordingly, the phenols formed were estimated from the change in absorption on going from acid to alkaline solution, at 290 mμ. for 3- and 4-chlorophenol, at 240 mμ. for 2-naphthol, and at 245 mμ. for 1-naphthol.

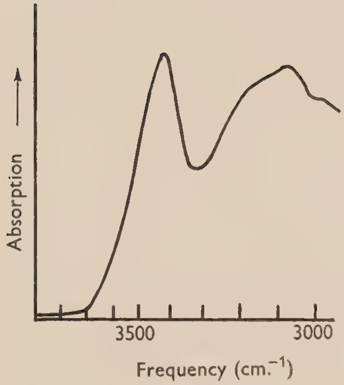

Fig. 2

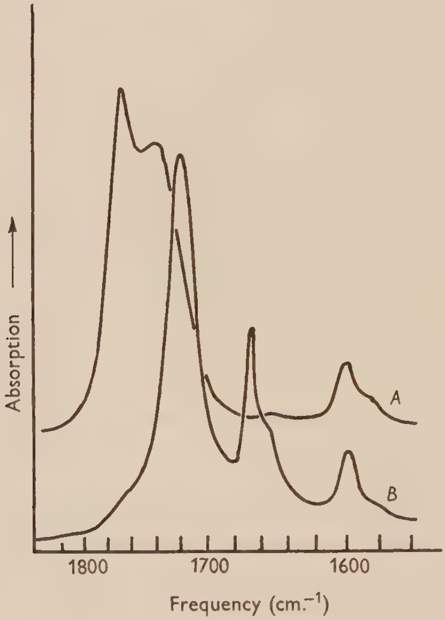

Fig. 3

Fig. 2. Absorption due to O—H stretching vibrations of β-hydroxy-γ-(2-methyl-4-chlorophenoxy)-butyric acid.

Fig. 3. Absorption due to C=O and C=C stretching vibrations of A, 2-methyl-4-chlorophenoxyacetic acid, and B, γ-(2-methyl-4-chlorophenoxy)-crotonic acid.

Paper chromatography. This was carried out generally as described previously (Webley *et al.* 1955). Occasionally the 'multiple development' technique of Jeanes, Wise & Dimler (1951) was used to obtain sufficient separation. It was particularly convenient with acids as it was unnecessary to use separate test strips to follow the resolution of the mixtures. The dried paper was sprayed with Universal Indicator solution (1:1 (v/v) mixture with water adjusted to pH 9·0 with dilute sodium hydroxide) and the position of the spots noted. The paper was then developed a second or third time. The indicator did not interfere. The solvent used by Byrde, Harris & Woodcock (1956) gave better separation of ω-naphthyl and ω-naphthyloxy substituted acids than the usual solvent (Fewster & Hall, 1951). For quantitative work, samples were dissolved in ethanol and a suitable amount corresponding to about 0·8 mg. total acids applied to the chromatogram. The position of the various acids was determined by using test strips. The strips containing the individual acids were immersed directly in 0·1% (w/v) sodium hydroxide solution (usually

5 ml.) for 15 min. at 50° and the decanted solution used for ultraviolet spectrometry. This technique was used to follow the β-oxidation of the chlorophenoxybutyric acids. The relative concentration of the components were estimated from their absorption ($e \doteqdot 1400$) at 275 to 280 mμ. Control experiments showed that a recovery of 95 % of the acid applied to the paper was to be expected.

Melting-points. These were determined with the Kofler heating stage.

RESULTS

The breakdown of the fatty acid side-chain of γ-phenyl- and γ-phenoxybutyric acids by nocardia strains

In contrast to phenylacetic acid, phenoxyacetic acid was not attacked by strain T_{16} but increases in oxygen uptake were obtained with phenoxybutyric and phenoxycaproic acids, presumably due to the breakdown of the fatty acid side-chain (Table 1). To confirm this a large-scale experiment was set up with phenoxybutyrate and T_{16} organisms as described in 'Methods'. Samples were removed aseptically after 17, 24, 41 and 48 hr. Paper chromatography of the extracts showed two spots in the early samples (17, 24 and 41 hr.) one corresponding to the original material. The latter had disappeared in the 48 hr. sample leaving only a substance with the R_F of phenoxyacetic acid. Material from the 41 hr. sample, dissolved in dioxan, showed the presence of both a β-hydroxy acid (about 30 %) and phenoxyacetic acid (about 25 %) by infrared analysis. It seems that the β-hydroxy acid formed from phenoxybutyric acid did not separate from phenoxyacetic acid on the paper chromatograms in contrast to the β-hydroxy acid formed from 3- and 4-chlorophenoxybutyric acid (Webley *et al.* 1957). Chromatographic examination of the samples failed to reveal the presence of phenols or phenolic acids.

Table 1. *The oxygen uptake of* Nocardia opaca *strain T_{16} in the presence of phenylacetic, phenoxyacetic, phenoxybutyric and phenoxycaproic acids*

Each vessel contained equiv. 15·3 mg. dry weight of a washed suspension of phenylacetate-grown *N. opaca*, 0·5 ml. 0·066 M-phosphate buffer (pH 7·2) and distilled water to 2·0 ml. After overnight incubation 0·5 ml. of distilled water or 0·02 M-sodium salts of the acids were tipped from the side arm.

Addition	O_2 uptake in 180 min. (μl.)
None	93
Phenylacetate	984
Phenoxyacetate	112
γ-Phenoxybutyrate	219
ε-Phenoxycaproate	558

The rates of breakdown of phenoxy- and phenylbutyric acids were compared with strain P_2, which does not attack phenylacetic acid (Webley *et al.* 1956). A preliminary manometric experiment suggested that the side-chain of phenylbutyric acid was broken down more easily than that of phenoxybutyric acid. This was confirmed by an experiment in which samples were removed after 24, 48, 65, 120 hr. Paper chromatography and subsequent examinations of the infrared spectra showed that phenylbutyric acid had been completely converted to phenylacetic acid by 24 hr. With phenoxybutyric acid conversion to a β-hydroxy acid (20%) and phenoxyacetic acid (40%) was found after 65 hr. (infrared spectra in dioxan). In a further experiment with 0·02 M-phenylbutyrate and strain P_2 organisms samples were taken at 8, 21, 31 and 48 hr. Those showing about 50% conversion by paper chromatography (8 and 21 hr.) were subjected to infrared analysis. No β-hydroxy acid absorption was detected in dioxan solution, indicating that this intermediate amounted to less than 10% of the total acids.

The metabolism of the fatty acid side-chain of ring-substituted ω-(phenoxy)-n-alkylcarboxylic acids by nocardia strains

Monochlorophenoxybutyric and caproic acids. Table 2 shows the oxygen uptake of strain T_{16} with phenoxybutyric acid and its 2-, 3- and 4-monochloro derivatives. Substitution in the 2-position brings about a marked reduction. A larger scale experiment was next set up with two sets of flasks containing 2-, 3- or 4-monochlorophenoxybutyric acids (sodium salts) and equal samples from a washed suspension of T_{16} organisms. After 7 days of incubation one set was examined by paper chromatography and infrared analysis. The 3- and 4-monochlorophenoxybutyric acids had been completely converted whilst 75% of the 2-chloro compound remained unchanged. After 14 days of incubation examination of the other set revealed that there was still 67% of the 2-chlorophenoxybutyric acid present. It was shown that these differences were not simply a reflection of the relative strengths of the acids (based on the pH value of half-neutralized equimolar solution of the acids in 50% aqueous ethanol).

Table 2. *The oxygen uptake of* Nocardia opaca *strain T_{16} in the presence of phenoxybutyric and* 2-, 3-, *and* 4-*monochlorophenoxybutyric acids*

Each vessel contained equiv. 7·1 mg. dry weight of a washed suspension of glucose-grown *N. opaca*, 0·5 ml. 0·066 M-phosphate buffer (pH 7·2) and distilled water to give 2·0 ml. After overnight incubation 0·5 ml. of distilled water or 0·05 M-sodium salts of the acids was tipped in from the side arm.

Addition	O_2 uptake in 180 min. (μl.)
None	59
γ-Phenoxybutyrate	121
γ-(2-Chlorophenoxy)-butyrate	82
γ-(3-Chlorophenoxy)-butyrate	118
γ-(4-Chlorophenoxy)-butyrate	124

The rates of conversion by T_{16} organisms of the 3- and 4-chloro compounds were also compared (Table 3). The 3-chloro compound was converted at a somewhat faster rate than the 4-chloro compound. The β-hydroxy acid intermediates formed during the conversion of these compounds have already been identified (Webley *et al.* 1957).

Comparison of the oxidation of 2-chlorophenoxycaproic and 2-chlorophenoxybutyric acids showed that only the former compound was actively metabolised. In a large-scale experiment paper chromatography showed that 2-chlorophenoxycaproic acid had been completely converted within 65 hr. to a substance with an R_F corresponding to 2-chlorophenoxybutyric acid while 2-chlorophenoxybutyric acid had remained unchanged.

Table 3. *The rate of conversion of* 3- *and* 4- *monochlorophenoxybutyric acids by* Nocardia opaca *strain* T_{16}

Flasks contained 0·0133 M-phosphate buffer (pH 7·2) and 0·004 M-3- or 4-monochlorophenoxybutyric acids (as sodium salt) and equiv. 231·6 mg. dry weight glucose-grown *N. opaca* to give final volume of 50 ml. Flasks were incubated on shaking machine at 25°, 25 ml. suspension removed aseptically after 3 days, and the remainder after 5 days. After centrifugation the supernatant fluids were extracted and quantitatively spotted on paper chromatograms. The acids were eluted separately and their concentration estimated from their absorption maxima ($e \doteqdot 1400$) at 275–280 mμ.

		Percentage of total products		
		Butyrate derivative	β-Hydroxy acid intermediate	Acetate derivative
γ-(3-Chlorophenoxy)-butyrate	3 days	40·5	59·5	—
γ-(4-Chlorophenoxy)-butyrate		77	23	—
γ-(3-Chlorophenoxy)-butyrate	5 days	22	67	11
γ-(4-Chlorophenoxy)-butyrate		51	49	—

Monochlorophenoxypropionic acids. The β-oxidation of phenoxypropionic acids might be expected to yield phenols (by way of the unstable phenylcarbonates, $C_6H_5.O.CH_2.CH_2.COOH \rightarrow C_6H_5.O.CO.OH \rightarrow C_6H_5.OH + CO_2$), and Fawcett, Ingram & Wain (1954) showed that plant tissue can in fact convert such compounds to phenols. The side-chain breakdown of the three monochlorophenoxypropionic acids by T_{16} organisms was followed by ultraviolet absorptiometry. Warburg flasks were set up containing 0·0133 M-phosphate buffer (pH 7·2), 0·002 M-2-, 3-, or 4-chlorophenoxypropionic acids (sodium salts), and the equivalent of 51·0 mg. dry weight of glucose-grown T_{16} organisms to give a final volume of 2·5 ml. in each vessel. After incubation at 25° for 18 hr., the flask contents were centrifuged and the supernatant liquid examined. No phenols were formed from 2-chlorophenoxypropionate, but 70 % of the 3-chloro- and 40 % of the 3-chlorophenoxypropionates had been converted to phenols, whose absorption agreed with the expected products. No attack on the benzene ring occurred under these conditions, but in experiments continued over long periods there were indications of the partial conversion of the 4-chlorophenol to polyhydroxyphenols, with absorption similar to 4-chlorocatechol. These results parallel closely those obtained from the monochlorophenoxybutyric acids.

Monomethylphenoxybutyric acids. The monomethylphenoxybutyric acids had a similar effect on the oxygen uptake of T_{16} organisms to the monochlorophenoxybutyric acids (Table 2). A large-scale experiment was set up with flasks containing 2-, 3-, or 4-monomethylphenoxybutyric acids (sodium salts) and T_{16} organisms. After 3 days of incubation samples from each flask were examined by paper chromatography; there had been virtually complete removal of the 3- and 4-methyl compounds but very little conversion of the 2-methyl compound. The infrared spectra of the solid products showed the formation of the acetate derivative from the 3- and 4-methylphenoxybutyric acids, although the yield from the latter was less than theoretical. Fresh organisms were added to each flask. The infrared spectra of the products at the end of the experiment showed that the 3- and 4-methylphenoxybutyric acids had now been completely converted to the corresponding acetate derivatives. The extracted material was recrystallized (twice) from benzene. The products from 3- and 4-methylphenoxybutyric acids had m.p. 104° and 139°, respectively, unchanged on admixture with the corresponding authentic acetates. The spectrum of the solid product from 2-methylphenoxybutyric acid indicated that the butyrate was largely unchanged.

Table 4. *Comparison of products from 3-methyl-, 3-chloro-, and unsubstituted-phenoxybutyric acids during β-oxidation by* Nocardia opaca *strain* T_{16}

Flasks contained 0·0133 M-phosphate buffer (pH 7·2) and 0·004 M-3-methyl- or 3-chloro- or unsubstituted-phenoxybutyric acids (sodium salts) and equiv. 374 mg. dry weight glucose-grown *N. opaca* to give a final volume of 100 ml. Flasks incubated at 25° for 42 hr. The ether-soluble products were examined in dioxan solution by infrared spectroscopy.

	Percentage of total products	
	β-Hydroxy acids	Substituted phenoxyacetic acid
γ-Phenoxybutyrate	45	25
γ-(3-Methylphenoxy)-butyrate	35	20
γ-(3-Chlorophenoxy)-butyrate	50	10

A manometric experiment suggested that a 2-chloro- had a greater effect than a 2-methyl-substituent on the β-oxidation of phenoxybutyric acid by *Nocardia opaca.* Confirmation of this was obtained by means of a large-scale experiment. Since the rate of conversion of the 2-substituted compounds is very slow it was necessary to make several additions of fresh organisms. Two culture flasks were set up containing 2-chlorophenoxybutyric or 2-methylphenoxybutyric acids and T_{16} organisms. Three further additions of fresh organisms were made to each flask. At the end of the experiment the infrared spectra of the products in dioxan showed that the 2-methyl- and 2-chlorophenoxybutyric acids had given 40 % and 15 % of the corresponding acetates, respectively; both samples contained about 30 % β-hydroxy acids. The results of a comparison of 3-methyl-, 3-chloro-phenoxybutyric acids, and the unsubstituted acid are given in Table 4. They indicate that the rate of conversion of the β-hydroxy intermediate is slower with the chlorine-substituted material. A comparison of 4-methyl- with 4-chloro-phenoxybutyric acids gave similar results.

The effect of di-substitution of the ring on the metabolism of the fatty acid side-chain of ω-phenoxyalkylcarboxylic acids by Nocardia opaca

Since substitution in position 2 (by methyl or chlorine) exerted such a marked effect on the rate of β-oxidation of the butyric acid side-chain of monosubstituted phenoxybutyric acids a few di-substituted compounds were tested. 2-Methyl-4-chlorophenoxybutyric acid (MCPB) and 2:4-dichlorophenoxybutyric acid (2:4-BD) are of special interest because of their practical importance as selective weed killers. It is known (Wain, 1956) that the herbicidal action of these substances is due to β-oxidation of the fatty acids side-chain within the plant to the corresponding acetate derivatives (MCPA or 2:4-DA) which are recognized herbicides. Flasks were set up with T_{16} organisms to compare 2-methylphenoxybutyric acid with MCPB. Two further additions of organisms were made and at the end of the experiment paper chromatography showed that there had been a much greater conversion of the 2-methyl compound than of the MCPB. Infrared analysis, in dioxan, showed 30% and 15% β-hydroxy acid intermediate from 2-methylphenoxybutyric acid and MCPB, respectively. The former sample contained 20% of the corresponding acetate derivative and the latter none. Similarly, when 3-chloro- and 4-chlorophenoxybutyric acids were compared with 3:4-dichlorophenoxybutyric acid it was found that the di-substituted compound was converted much more slowly than the mono-substituted ones. These experiments clearly demonstrated that di-substitution decreases the rate of β-oxidation. MCPB and 2:4-DB were next compared. Two flasks containing MCPB or 2:4-DB and T_{16} organisms were set up in the usual way, and three further additions of fresh organisms were made. At the end of the experiment paper chromatography revealed that MCPB and 2:4-DB had been converted 50% and 20%, respectively. Infrared examination of the products in dioxan solution showed that a β-hydroxy acid was produced in each case; MCPA and 2:4-DA were not detected. In a similar experiment, but with seven additions of T_{16} organisms chromatography showed that the original material had almost all been converted to an unknown substance. Infrared examination after recrystallization of the material from benzene showed this to be (by comparison with a synthetic specimen) the β-hydroxy derivative of MCPB. In previous work (Webley *et al.* 1957) it had been found possible to convert further the β-hydroxy acid formed from 3- and 4-monochlorophenoxybutyric acids to the corresponding acetate derivatives. Attempts to bring about a similar conversion of the β-hydroxy acid of MCPB were unsuccessful. To see whether 2-methyl-4-chlorophenoxycrotonic acid could be converted to the β-hydroxy acid by the organism a culture flask was set up containing a 0·001 M solution of this compound and T_{16} organisms. Five further additions of fresh organisms were made. Evaporation of the ether extract obtained at the end of the experiment gave an oil, which on standing crystallized. After recrystallization from aqueous ethanol the infrared spectrum of the product agreed closely with that of authentic β-hydroxy-γ-(2-methyl-4-chlorophenoxy)-butyric acid.

In view of the powerful effect of di-substitution on the β-oxidation of the side-chain of phenoxybutyric acid it was thought worthwhile to see whether a similar effect operated with a longer side-chain acid. 2-Methyl-4-chlorophenoxycaproic (MCPC) and 2:4-dichlorophenoxycaproic (2:4-DC) acids gave marked increases in oxygen uptake (Table 5) when compared with MCPB and 2:4-DB, suggesting a much more rapid breakdown of the side-chain. This was confirmed in an incubation experiment with MCPC and washed T_{16} organisms, where it was found that one further addition of organisms was sufficient to convert MCPC to MCPB in 3 days.

Table 5. *The oxygen uptake of* Nocardia opaca *strain* T_{16} *in the presence of MCPC, MCPB, MCPA, 2:4-DC and 2:4-DB*

Each vessel contained equiv. 8·4 mg. dry weight of a washed suspension of glucose-grown *N. opaca*, 0·5 ml. 0·066 M-phosphate buffer (pH 7·2) and distilled water to give 2·0 ml. After overnight incubation 0·5 ml. of distilled water or 0·005 M-sodium salts of the acids were tipped in from the side arm.

Addition	O_2 uptake in 180 min. (μl.)
None	60
MCPC	210
MCPB	84
MCPA	60
2:4-DC	189
2:4-DB	78

The breakdown of the fatty acid side-chain of ω-(1-naphthyl)-, ω-(1-naphthyloxy)-, and ω-(2-naphthyloxy)-butyric and -propionic acids by Nocardia opaca

γ-(1-naphthyloxy)- and γ-(2-naphthyloxy)-butyric acids. Byrde *et al.* (1956) showed that γ-(2-naphthyloxy)-butyric acid underwent β-oxidation by *Aspergillus niger* after hydroxylation of the naphthyl nucleus, the final product being (6-hydroxy-2-naphthyloxy)-acetic acid. They also showed that an intermediate was formed between γ-(6-hydroxy-2-naphthyloxy)-*n*-butyric acid and (6-hydroxy-2-naphthyloxy)-acetic acid but did not attempt to isolate or identify this compound. The breakdown of γ-(1-naphthyloxy)- and γ-(2-naphthyloxy)-butyric acids by the soil nocardias used here was investigated. Flasks were set up containing 0·002 M solutions of these compounds and T_{16} organisms. Since only about 20% conversion of the 2-naphthyloxy compound had taken place after 8 days two further additions of fresh organisms were made. Ether extracts from samples taken immediately before the last addition showed that the product from γ-(2-naphthyloxy)-butyric acid was principally the β-hydroxy acid intermediate, by comparison of its infra-red spectra with that of an authentic specimen, prepared from the corresponding ester by the method of Grün (1925). Seven days after the third addition of organisms the γ-(2-naphthyloxy)-butyric acid was found to be completely converted to the corresponding acetate (m.p. and mixed m.p. 154° after two recrystallizations from benzene). In contrast, γ-(1-naphthyloxy)-butyric acid was unchanged throughout a similar experiment. Phenols or phenolic acids could not be detected in either case, showing that *Nocardia opaca*, strain T_{16}, unlike *A. niger* cannot hydroxylate the naphthyl nucleus.

β-(1-naphthyloxy)- and β-(2-naphthyloxy)-propionic acids. When the side-chains of the above compounds are metabolized by β-oxidation the expected products would be 1-naphthol and 2-naphthol respectively (cf. monochlorophenoxypropionic acids). The following experiment was carried out to follow the production (if any) of phenols. Two flasks containing 0·001 M-β-(1-naphthyloxy)- or β-(2-naphthyloxy)-propionic acids (sodium salts) and T_{16} organisms were set up and sampled after 0, 4, 6 and 8 days. Ultraviolet spectrometry after removal of organisms showed that 38 % of the β-(2-naphthyloxy)-propionic acid was converted to 2-naphthol in 6 days, with no further change in 8 days. No naphthol was formed from β-(1-naphthyloxy)-propionic acid and in neither case was there any attack on the ring. After 12 days the experiment was ended and the extracted products examined by paper chromatography. The extract from the 2-naphthyloxy compound contained a substance with an R_F value corresponding to 2-naphthol. No phenols or phenolic acids were present in the material from the 1-naphthyloxy experiment. The results of this experiment parallel the previous observations with γ-(1-naphthyloxy)- and γ-(2-naphthyloxy)-butyric acids, showing the marked difference in the β-oxidation of the fatty acid side-chain depending on whether it is attached to position 1 or position 2 of the naphthyl nucleus through an oxygen bridge.

γ-(1-naphthyl)- and γ-(1-naphthyloxy)-butyric acids. Phenylbutyric acid was metabolized more rapidly than phenoxybutyric acid (see Table 2), but the difference between 1-naphthyl- and 1-naphthyloxy-butyric is even more striking as was demonstrated in the following experiment. T_{16} organisms were added to two flasks containing 0·002 M γ-(1-naphthyl)- or γ-(1-naphthyloxy)-butyrate. Analysis of samples taken after 7 days showed that 1-naphthylbutyric acid had been completely converted to the acetate, whereas the 1-naphthyloxybutyric acid was unchanged. Further addition of organisms had no effect on the latter compound.

The breakdown of the fatty acid side-chain of γ-phenyl-, γ-(3-indolyl)- and γ-(1-naphthyl)-butyric acids by Nocardia *sp. strain P_2*

The result of a manometric experiment suggested that the fatty acid side-chains of phenyl- and indolyl-butyric acids are oxidized more rapidly than that of γ-(1-naphthyl)-butyric acid. This was confirmed in an incubation experiment with these compounds set up in separate flasks with equal samples from the same washed suspension of strain P_2 organisms. Examination of 24 hr. samples revealed that whereas the phenyl- and indolyl-butyric acids had been completely converted to their corresponding acetate derivative the γ-(1-naphthyl)-butyrate remained practically unchanged. In another experiment with phenyl- and indolyl-butyric acids in which samples were examined at shorter intervals it was found that in 3 hr. there had been complete conversion of phenylbutyric acid, whereas after 7 hr. indolylbutyric acid was still only *c.* 50 % converted.

Occurrence of β-hydroxy- and αβ-unsaturated intermediates during β-oxidation by strain T_{16} and P_2

With the techniques used in an earlier study of the β-oxidation of β-phenylpropionic acid to benzoic acid it was shown that cinnamic acid was an intermediate (Webley *et al.* 1955), but as small amounts of the β-hydroxy intermediate would probably not have been detected, this system has been re-investigated. Samples were taken for analysis at 8, 24, and 48 hr. after addition of strain P_2 organisms to 0·02 M-phenylpropionate. The infrared spectra (dioxan solution) of the mixture of acids extracted from samples showing partial conversion to benzoic acid (8 and 24 hr.) had no detectable absorption arising from alcoholic hydroxyl groups, indicating that the β-hydroxy intermediate, if present, did not exceed 10 %. Cinnamic acid (about 10 %) was clearly in evidence (C═C absorption at 1640 cm.$^{-1}$). These observations were confirmed with T_{16} organisms in a similar experiment. In contrast, during the β-oxidation of the variously substituted phenoxybutyric acids, the β-hydroxy acid intermediates have been detected in amounts between 25 and 75 % of the total acids, but no αβ-unsaturated intermediates are detectable (i.e. less than 5 % present). Neither β-hydroxy nor αβ-unsaturated acids have been found during β-oxidation of phenylbutyrate by P_2.

DISCUSSION

The following factors affect the β-oxidation of the fatty acid side-chain of ω-aryl- and ω-aryloxy-*n*-alkylcarboxylic acids by washed suspensions of the nocardias used in the present work. (*a*) An oxygen bridge between the fatty acid and the ring; as with phenylbutyric and phenoxybutyric and with γ-(1-naphthyl)butyric and γ-(1-naphthyloxy)butyric acids. (*b*) Ring substitution, particularly in the 2-position. (*c*) Nature of the ring substituents (e.g. chlorine and/or methyl groups). (*d*) Kind of ring structure to which the fatty acid is attached (phenyl, indolyl and naphthyl). (*e*) Position of attachment of the fatty acid; as with ω-(1-naphthyloxy)- and ω-(2-naphthyloxy)-propionic and -butyric acids. The effects of (*a*), (*b*) and (*c*) are most marked when the side-chain is short as in propionic or butyric acids, and although not tested this is probably true of (*d*) and (*e*).

A simple theory based on the steric and electronic (inductive) effects of the substituents can only give a partial explanation for all the observations in the present work. It is now clear that the appearance of β-hydroxy acid intermediates is common to all the ω-aryloxybutyric acids, and is not solely an effect of chloro-substituents as previously suggested in work on 3- and 4-chlorophenoxybutyric acids (Webley *et al.* 1957). Our present work, however, repeatedly shows that the presence of a chlorine substituent appears to delay the conversion of the β-hydroxy acid intermediate to the acetic acid derivative leading to a greater accumulation of the former. This may be due to an inductive effect of the chlorine atom reinforcing the tendency of the ether oxygen to withdraw electrons from the side-chain. Further, while steric hindrance may be adequate to account for the slower oxidation of β-(1-naphthyl)-propionic (Webley *et al.* 1956), and γ-(1-naphthyl)-butyric acid compared with the corresponding phenyl derivatives, it seems inadequate to account for the big difference in the rates of β-oxidation of γ-(1-naphthyloxy)- and γ-(2-naphthyloxy)-butyric acids. Clearly work on isolated enzymes (cf. Stern & del Campillo, 1955) involved in the various stages would be necessary to provide fuller explanations. The results obtained with MCPB and 2:4-DB are of interest since these compounds are well-known herbicides; the principle on which they operate has been described by Wain (1955) who suggested that two different plants might respond differently to these compounds because the β-oxidizing enzymes were unable to operate in the unaffected plant. The present work suggests, by analogy, that the β-oxidizing system of the unaffected plant may be affected to a greater extent by substitution than that of the sensitive plant.

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The Growth of *Brucella abortus* Strain 19 in Aerated Dialysed media

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SUMMARY: An apparatus for growing *Brucella abortus* in a dialysed medium in an aerated cellophan tube is described from which 500 ml. of culture of strain 19 having a density of 7 to 10×10^{11} organisms/ml., equivalent to 50–70 g. dry weight of organisms/l. could be harvested every 3 days.

The production of vaccine from *Brucella abortus* strain 19 grown on the surface of solid media is extremely costly because of the high rate of contamination, and of the low yield. Little interest has been shown in deep culture methods for the routine production of Brucella vaccine in spite of promising experimental results by a number of workers (Gerhardt & Gee, 1946; Glassman & Elberg, 1946; Saunders & Huddleson, 1950). Sterne & Wentzel (quoted by van Drimmelen, 1952) obtained *c.* 150×10^9 viable *Brucella abortus*/ml. in shake flasks in volumes of 3–4 l. This method was further developed by van Drimmelen (1956) and is used in South Africa for routine vaccine preparation. Dr G. P. Gladstone in 1947 (personal communication) obtained large harvests of *Salmonella typhi* in media aerated in cellophan tubes, and later (Gladstone, 1948) used a similar technique for preparing anthrax immunizing antigen. Gorelik, Mead & Kelly (1951) showed that the growth of *Brucella suis* in shake flasks could be increased by enclosing the medium in cellophan sacs. Anderson (1953, 1954, 1955) at the South African Institute for Medical Research has obtained yields of *S. typhi* up to $1{\cdot}2 \times 10^{11}$ organisms/ml. in aerated cellophan tubes, and Dr J. H. Mason of the same Institute suggested that the method should be tried for growing *B. abortus*. The apparatus described below is virtually identical with that used by Fredette & Vinet (1952) for growing *Clostridium tetani*. It is simple to operate and has yielded up to $1{\cdot}8 \times 10^{12}$ viable organisms/ml. with *B. abortus* strain 19.

METHODS

Apparatus. Figure 1 shows a drawing of the apparatus used. The culture vessel K is 780 mm. long and 80 mm. diam. The constricted ends H and H_1 are 48 mm. diam. and 40 mm. long. Tubes I_1 and I are for inflow and outflow of medium respectively. L is a cellophan tube (Visking's regenerated cellulose tubing, diam. $1\frac{7}{8}$ in.). This tubing is well soaked in water, threaded through K, and the ends reflected over H and H_1 where they are held in position by rubber pads (25 mm. thick) F and F_1 clamped between metal disks E and E_1 by means of three equally spaced $\frac{1}{4}$ in. threaded metal rods B furnished with wing nuts; only two of these rods are shown in the figure. M is a sparger formed of a 40 mm. no. 3 sintered disk which is connected to an air supply through a humidifier and a filter (not shown). *N* is a pyrex Y-piece, one branch of which is connected to a reservoir containing physiological saline, while the other branch is used for sampling and harvesting the culture and for steaming the apparatus (see below). *A* is a 500 ml. boiling flask with a tube *C*, 25 mm. diam., fused into the bottom. This is connected to a 25 mm. diam. tube *D* which pierces *F*. *G* is a stainless steel spiral to prevent deformation of the cellophan tube *L* during aeration.

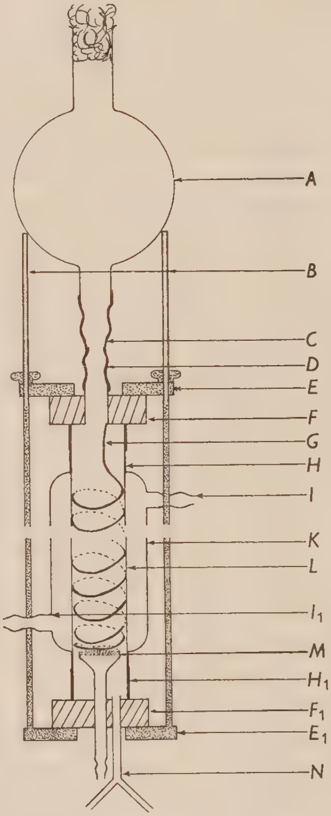

Fig. 1. Apparatus for deep culture of *Brucella abortus*.

The apparatus is assembled as shown and furnished with appropriate rubber connexions for attachment to the reservoirs for medium and saline, the air-line, and a reservoir for spent medium. About 50 ml. distilled water are introduced into the cellophan tube *L*, the wing nuts tightened sufficiently to make a good seal between the ends *H*, the cellophan tube *L* and the rubber pad *F*, and the whole apparatus then autoclaved. After sterilization, the apparatus can be left for long periods, assembled and ready for use, without risk of contamination.

Medium. The culture medium used was virtually the same as that suggested by van Drimmelen (1952): 3% (w/v) ‘peptone’ (the peptone was a papain digest of horse muscle containing 17 g. total-N/100 g.); 3% (w/v) glucose; 1% (w/v) Marmite; 0·15% (w/v) anhydrous Na_2HPO_4. The pH was adjusted to 6·4 and the medium sterilized by filtration through Seitz pads.

Operation of the apparatus. The cellophan tube *L* is filled through *N* with *c.* 1000 ml. physiological saline from a reservoir and the surrounding annular space filled with *c.* 1500 ml. medium from another reservoir through I_1. After diffusion has proceeded for about 4 hr. the diffusate in *L* is inoculated with the washings of an agar slope culture of *Brucella abortus* strain 19 together with antifoam (Dow-Corning silicone antifoam A in technical light oil) and air is passed through at the rate of 250 ml./min. The whole apparatus is incubated in a hot room at 37° C. After 24 hr. growth a drip of 400 ml. of medium/24 hr. is started. The culture is harvested after 3–4 days, the cellophan tube then refilled with saline and fresh medium run into the annular space. Growth is more rapid in the second and subsequent cycles because of the large residue of actively growing organisms left from the previous run.

In order to avoid the development of mutants, the apparatus is steamed after four to six cycles by attaching a wash bottle of sterile distilled water to the harvesting nozzle *N* and boiling the water over a gas ring. In practice, there has been little tendency for rough variants to appear so long as there is no interruption to the air supply.

RESULTS

The results of eight successive batches are given in Table 1. Clearly, the number of organisms/ml. varied inversely as the volume harvested. This was due to concentration by dialysis since there was virtually no increase in the total number of viable organisms between 72 and 96 hr. An average expectation was a harvest of 500 ml. containing 7 to 10×10^{11} viable organisms/ml. equivalent to 50–70 g. dry wt. organisms/l. The average total harvest was *c.* 400×10^{12} viable organisms/batch. The fluctuation in the proportion of viable

Table 1. *Details of eight successive batches* (28 *harvests*) *of* Brucella abortus strain 19 *grown in aerated dialysed medium*

Batch	Cycle	Volume harvested (ml.)	Opacity by Brown's tubes	Yield of organisms (equivalent g. dry weight/l.)	Total yield of organisms in harvest (equivalent g. dry weight)	No. of viable organisms/ml. harvest ($\times 10^9$)	Total no. viable organisms in harvest ($\times 10^{12}$)	No. viable organisms/g. dry weight organisms ($\times 10^{12}$)
4	1	850	350	28·9	24·6	496	422	17·2
	2	750	450	45·8	34·4	—	—	—
	3	400	800	77·2	30·9	1015 ± 32	406 ± 13	13·1 ± 0·4
5	1	510	350	36·5	18·6	448	228	12·3
	2	350	650	67·9	23·8	960 ± 64	336 ± 23	14·1 ± 1·0
	3	425	500	54·6	23·2	640 ± 48	272 ± 21	11·6 ± 0·9
	4	600	400	34·7	20·8	547 ± 48	328 ± 29	15·8 ± 1·4
	5	600	500	48·2	28·9	1080 ± 90	648 ± 54	22·4 ± 1·9
6	1	375	600	72	27	1156 ± 56	434 ± 21	16 ± 0·8
	2	500	500	43·9	22	509 ± 18	255 ± 9	11·6 ± 0·4
	3	500	640	—	—	824 ± 58	412 ± 29	—
	4	370	700	76·3	28·2	1021 ± 54	378 ± 20	13·4 ± 0·7
	5	580	350	29·5	17·1	562 ± 32	325 ± 19	19·0 ± 1·1
	6	350	750	75·9	26·6	1616 ± 93	565 ± 33	21·2 ± 1·2
7	1	500	520	58·3	29·2	858 ± 48	429 ± 24	14·7 ± 0·8
8	1	550	320	34·7	19·1	536 ± 39	295 ± 22	15·4 ± 1·1
	2	600	300	38·2	22·9	389 ± 32	233 ± 19	10·2 ± 0·9
9	1	460	450	48·9	22·5	691 ± 43	318 ± 20	14·1 ± 0·9
	2	460	500	49·4	22·8	698 ± 45	321 ± 22	14·1 ± 0·9
	3	500	480	46·2	23·1	909 ± 38	455 ± 19	19·7 ± 0·8
	4	420	500	50·1	21	811 ± 43	341 ± 18	16·2 ± 0·9
10	1	530	420	40·4	21·4	749 ± 58	397 ± 32	18·5 ± 1·5
	2	430	630	57·7	24·8	1280 ± 141	550 ± 52	22·2 ± 2·1
	3	455	480	45·4	20·7	883 ± 64	393 ± 29	18·9 ± 1·4
	4	430	500	54	23·2	672 ± 58	289 ± 25	12·5 ± 1·1
11	1	600	250	—	—	—	—	—
	2	450	540	64	28·8	970 ± 74	437 ± 34	15·1 ± 1·2
	3	240	1200	117	28·1	1797 ± 168	431 ± 40	15·3 ± 1·4

organisms (column 9) was more apparent than real and reflected mainly variations in the efficiency of the medium on which the viable counts were made.

A harvest of 400×10^{12} organisms makes 6000–7000 doses of vaccine, and an average of six cycles/month can be maintained comfortably, so that 400,000 to 500,000 doses/year can be produced by one such apparatus which, with all its accessories, occupies only 4·5 sq.ft. of incubator floor space. The labour required is a fraction of that needed for producing similar amounts of vaccine by conventional methods.

I am indebted to Dr J. H. Mason of the South African Institute for Medical Research for stimulating interest in this method, and to Dr Fredette for suggesting the very effective method of clamping the cellophan. I should also like to acknowledge the valuable help given by Mr I. Hutchinson and Mr D. Wetherby of the Wellcome Research Laboratories.

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Reversible Changes in Strains of Tobacco Mosaic Virus from Leguminous Plants

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SUMMARY: Strains of tobacco mosaic virus (TMV) obtained from systemically-infected leguminous plants in Nigeria and India changed their properties greatly when propagated in different hosts. From systemically-infected tobacco, they closely resemble type TMV and share many antigens with it, but from systemically-infected French bean, they differ from it by at least as much as any previously described strains. The different forms of the viruses share few antigens, have different amino acid constitutions, electrophoretic behaviour and resistance to inactivation by ultraviolet radiation, and they produce different kinds of local lesion in *Nicotiana glutinosa*. Isolates derived from single local lesions behave like the original bulk cultures, and as the changes are also reversible they seem unlikely to occur simply because different hosts select different strains from existing mixtures. The electrophoretic patterns, particularly of preparations from beans, show that infected plants contain more than one anomalous product; both forms of the viruses may be produced in all hosts, but there is no evidence that sap from systemically-infected beans contains the tobacco forms in amounts needed to cause infection. The change after transfer to a new host apparently occurs because mutants produced in that host are favoured over the infecting form because they move more readily and become systemic. The bean forms multiply more extensively in tobacco at above 30° than at 20° and above 30° seem not to produce forms that give a systemic mosaic; many tobacco plants at 20° develop only local lesions, and systemic infection, indicating the occurrence of the tobacco forms, happens more often in young seedlings than when mature plants are inoculated.

Some varieties of French bean (*Phaseolus vulgaris* L.) produce discrete necrotic local lesions when infected with tobacco mosaic virus (Price, 1930; Silberschmidt & Kramer, 1941) and are often used to assay virus preparations. There seems to be no record of any other kind of symptom caused by tobacco mosaic virus in leguminous plants until Lister & Thresh (1955) reported that a systemic disease of cow-pea (*Vigna unguiculata* (L.) Walp.) and Bengal bean (*Mucuna aterrima* Holland) in Nigeria was caused by a strain of this virus, which also caused systemic symptoms in the fourteen varieties of French bean they inoculated. Bawden (1956) confirmed that this disease is caused by a strain of tobacco mosaic virus and reported that, as obtained from systemically-infected beans, this strain differs greatly in its constitution and properties from when obtained from systemically-infected tobacco plants. He also reported that southern sann-hemp mosaic in India (Capoor, 1950) is caused by a strain of tobacco mosaic virus, which also undergoes comparable changes when transferred from leguminous to solanaceous plants. The present paper describes some of the unusual behaviour of these virus strains.

METHODS

Most of the work was done with a virus culture from cowpea kindly supplied by Mr R. M. Lister from Nigeria; this will be called the cowpea virus and, for convenience, as obtained from systemically-infected leguminous plants, it will be referred to as the bean form and from systemically-infected tobacco plants as the tobacco form. The two forms of the southern sann-hemp virus, which was kindly supplied by Dr S. P. Capoor, will be similarly distinguished.

The French bean variety mostly used was Prince, which gives neither local nor systemic symptoms with the type strain of tobacco mosaic virus, but Pinto beans, which give necrotic local lesions and no systemic symptoms with the type strain, were sometimes inoculated. Inoculations were usually made to the first-formed true leaves, at the time the first trifoliate one was unfolding. The tobacco (*Nicotiana tabacum* L.) was Judy's Pride, a variety of the White Burley type. Local lesion assays with both forms of the two viruses were made on *N. glutinosa*. Unless otherwise specified, all plants were raised and kept in a glasshouse heated during winter, with no supplementary lighting and with temperature fluctuating around a mean of 20°. Except when doing assays, leaves to be inoculated were usually lightly dusted with 'Celite', a diatomaceous earth that facilitates transmission, before they were rubbed over their upper surfaces with the fore-finger wetted with inoculum (usually undiluted sap) and then rinsed in a stream of water.

Antisera were prepared by injecting rabbits intravenously with purified virus preparations. Precipitation tests were usually made with antiserum at a constant dilution; 1 ml. of suitably diluted antiserum was added to each of a series of tubes containing 1 ml. antigen solution at different concentrations. The tubes were placed in a glass-fronted water bath at 50°, with the fluid columns half immersed to ensure continuous mixing, and illuminated from behind so that the formation of precipitates could be continuously observed.

Purified virus preparations were made by precipitating the virus from clarified sap successively with ammonium sulphate and acid. The sap was usually clarified by heating to 58° and low-speed centrifugation, and after precipitation with salt and acid the partially purified preparations were incubated overnight at 36° with 0·1 M-sodium citrate at pH 7. They were then clarified, the virus precipitated again with ammonium sulphate and acid, resuspended at pH 7, sedimented by ultracentrifugation, taken up in and dialysed against distilled water. Preparations of the bean form were less easy to purify than were preparations of the tobacco form, and the bean form also went into solution less readily after precipitation with salt or acid. Salt-free preparations of both forms remained clear indefinitely and when more concentrated than about 20 g./l. they usually produced a liquid crystalline layer when left undisturbed. Originally clear solutions of the bean form when kept in 0·1 M-phosphate buffer (pH 7) became milky within a few days at 18° and more slowly at 0°. Left undisturbed in this medium the virus settled out as a white fibrous precipitate, which in some ways resembles that produced from preparations of the type strain of tobacco mosaic virus with nicotine and salt

(Bawden & Pirie, 1940). A difference, though, is that vigorous shaking does not disperse the fibres of the bean form to give a clear fluid. Preparations of the tobacco form, like those of other strains of tobacco mosaic virus, do not become insoluble in phosphate buffer. In the insoluble state produced by phosphate, the bean form does not move in an electric field at pH 7.

Electrophoretic measurements were made in a Perkin-Elmer apparatus, model 38 A, with the Philpot-Svenson optical system, and a potential gradient of 9·5 V./cm.

RESULTS

Symptoms in bean, tobacco and Nicotiana glutinosa

The symptoms produced by the cowpea virus in Prince beans vary with time of the year, but Pl. 1, figs. 1 and 2, give an idea of the most usual effects. Plate 1, fig. 1 is a photograph of a first-formed leaf that was inoculated from systemically-infected beans and shows the many local lesions that often develop; these are white or yellow, superficially necrotic, and give the appearance of etched rings or patterns. Such local lesions do not occur when beans are inoculated with the tobacco form of the virus, and the inoculated leaves remain symptomless. Systemic symptoms are similar whether the inoculum comes from systemically-infected bean or tobacco plants, but many plants inoculated from tobacco fail to show symptoms and those that do take 5–7 days longer to do so than plants inoculated from beans. The first systemic symptoms are usually a clearing of the veins of the young trifoliate leaves, which may also then become bronzed and wilt slightly. Later-formed leaves show a mosaic of light and dark green areas, and as the length of infection increases so the newly produced leaves show increasingly obvious symptoms; in addition to mottling, they may show large blisters and other deformities, the laminae may be so decreased that little remains except the main veins; many leaves also produce enations, abnormal outgrowths from the main or subsidiary veins (Pl. 1, fig. 2).

When inoculated with the bean form, Pinto beans behave like Prince beans and give similar systemic symptoms, but when inoculated with the tobacco form they give dark necrotic local lesions, resembling those caused by the type strain of tobacco mosaic virus (TMV); these are the only symptoms and the uninoculated leaves remain normal in appearance and free from virus.

When first obtained from India the sann-hemp virus produced a milder disease in Prince beans than that caused by the cowpea virus, but after propagation for 2 years in the glasshouses at Rothamsted it has increased in virulence and both viruses now cause similar symptoms. This is unlikely to be because of contamination with the cowpea virus, for whereas the cowpea virus has also changed in that it has become increasingly difficult to transfer it from bean and get systemic infection in tobacco, the bean form of the sann-hemp virus still produces systemic infection in tobacco as readily as at first.

Both the cowpea and sann-hemp viruses cause systemic symptoms in tobacco that closely resemble those caused by type TMV, a mottling in various shades of green, with occasional yellow or necrotic spots; the growth of the plant and size of leaf is decreased and the leaves are also often somewhat deformed. The only way of diagnosing whether infection is by type TMV or the tobacco forms of the cowpea or sann-hemp viruses is to inoculate bean plants and see whether they become systemically infected. Tobacco leaves inoculated with the tobacco forms of these viruses sometimes, as with type TMV, produce faint chlorotic spots, but usually they show no local lesions. Tobacco leaves inoculated with the bean form usually produce local lesions, but their distinctness and type vary; sometimes they are brown necrotic spots, sometimes chlorotic with minute necrotic centres. Many plants produce lesions only on the inoculated leaves, but others develop systemic symptoms of the mosaic type, which also take 5–7 days longer to appear than in plants inoculated with the tobacco forms, even when the inocula of the tobacco forms are diluted almost to the infection end-point.

Whether the bean form of the cowpea virus produces a systemic infection depends greatly on the age and condition of the tobacco plants when they are inoculated. The chances of it doing so decrease as the age of the inoculated plant increases and as its rate of growth decreases; they are greatest with young rapidly growing plants with 3–5 leaves 2–3 in. long. When the virus was first obtained from Nigeria, undiluted sap from systemically-infected beans, inoculated to such plants with the addition of 'Celite', always infected all of them systemically, and it infected about half of those which were older and had leaves 6–10 in. long. Now systemic infection is more difficult to get; it rarely occurs in the older type of plant and more than half of inoculated young seedlings also often show local lesions only. The tobacco form gives systemic infections, apparently with equal readiness, both in these kinds of tobacco plants and in ones much older and growing more slowly.

The symptoms caused by the bean forms of the cowpea and sann-hemp viruses in tobacco depend on the temperature at which the inoculated plants are kept. Those symptoms already described relate to ordinary glasshouse conditions with a mean temperature of 20°. At constant temperatures of 30–35° there are no symptoms, either local or systemic; despite this the virus multiplies and reaches larger amounts in such symptomless inoculated leaves than when the plants are kept at lower temperatures and show lesions. When, after a week or so at over 30°, the plants are put at ordinary glasshouse conditions, the inoculated leaves develop large necrotic rings or spots. When kept for longer at 30°, occasional necrotic spots may also occur on uninoculated leaves after the plants are returned to ordinary glasshouse conditions, but there is no general systemic infection; such plants rarely produced mosaic symptoms when maintained in the glasshouse for long periods, although a few did after 2 weeks or more. It seems that, at the higher temperatures, the bean form multiplies as such in inoculated tobacco leaves and occasionally moves from these, but it becomes established in only a few isolated areas where it again multiplies without producing any of the tobacco form. The tobacco form, although it may not reach such high concentrations in tobacco plants kept at 36° as at lower temperatures, readily multiplies in them, systemically invades them, and causes mosaic symptoms.

In *Nicotiana glutinosa* the cowpea and sann-hemp viruses both behave similarly, but in their different forms produce very different lesions. The tobacco forms behave like type TMV. Under ordinary glasshouse conditions, local lesions appear in about 48 hr. and gradually increase in size. Their size and character varies with the age of the leaf inoculated and with the state of growth of the plants and all those on one leaf are not uniform, but in their most usual form they reach a diameter of 3–4 mm. and are clearly defined with a light centre surrounded by a ring of dark-brown or black (Pl. 1, fig. 3). The lesions produced by the bean forms of the viruses do not become visible until about 4 days after inoculation and they are much smaller and less well defined; they also vary with the age and condition of the plant, but few reach a diameter greater than 1 mm., and although some have partial rings of black, most are simply white flecks (Pl. 1, fig. 3). The differences between the lesions are large enough easily to distinguish the presence of small amounts of the tobacco forms in the presence of much of the bean forms.

In *Nicotiana glutinosa* also the results of infection depend on the temperature at which the inoculated plants are kept. At usual temperatures infection by both forms is restricted to the local lesions. At 36° the tobacco form, like type TMV, causes large chlorotic local lesions (Pl. 1, fig. 4) and a systemic mottling; when plants with these symptoms are placed at 20°, the chlorotic areas soon become necrotic. At 30–36° the bean form multiplies in inoculated *N. glutinosa* leaves and reaches greater amounts than at 20° but, as in tobacco, causes no symptoms (Pl. 1, fig. 4); the areas in which the virus multiplies become necrotic soon after plants are placed at 20°.

In the necrotic lesions formed on *Nicotiana glutinosa* kept and infected under normal glasshouse conditions, the tobacco forms of both the cowpea and the sann-hemp viruses seem to multiply more extensively than the bean forms, for when single lesions are cut out, macerated and used to inoculate other *N. glutinosa*, many infections are obtained from lesions caused by the tobacco forms and few from those caused by the bean forms of the virus. However, this conclusion is not necessarily correct, because weight for weight the tobacco forms of the viruses are considerably more infective towards *N. glutinosa* than are the bean forms. To quote one comparison with purified preparations of the cowpea virus, in which opposite half leaves were rubbed with the two forms at concentrations of 100, 10, 1 and 0·1 mg./l., the bean form produced average numbers of lesions/half leaf of 35, 16, 4 and 0·1 and the tobacco form produced 60, 34, 12 and 5. That this difference was not produced by any extra inactivation of the bean form during the processes of purification was shown by tests with clarified sap, when again sap from systemically-infected tobacco plants was ten or more times as infective per unit of virus as measured serologically as sap from systemically-infected beans. The virus content of sap from both tobacco and bean leaves varies with the condition of the plants and the time they have been infected, but tobacco sap usually contains 5–10 times more than bean sap. Hence, in comparisons with sap at different dilutions, similar numbers of lesions are produced by the two when tobacco sap is diluted about 100 times more than bean sap.

From single lesions produced in *Nicotiana glutinosa*, ten separate cultures of the cowpea virus were established, six produced by virus coming from tobacco and four from bean. Each culture was transferred serially four times in *N. glutinosa* and each time the inoculum for the next *N. glutinosa* was derived from one local lesion well separated from any other. The type of lesion remained constant during these transfers, the virus originating from bean producing each time only a few, small, slowly developing lesions and that originating from tobacco producing many more, larger and rapidly developing ones. After the fourth such transfer, sap from *N. glutinosa* infected from single lesions was used to inoculate bean and tobacco plants. Of the 6 cultures derived from the tobacco form, all immediately infected tobacco systemically and 2 bean; the 4 that did not immediately infect bean did so when inoculations were later made from the tobacco plants infected with them. Similarly, of the 4 cultures derived from the bean form, all infected bean directly from *N. glutinosa* and only 1 gave a systemic infection in tobacco, but subsequent inoculations from all the infected beans to tobacco gave systemic infections. The subsequent behaviour of none of these single-lesion cultures differed from that of the original bulk culture, and each when transferred from bean to tobacco underwent comparable changes in properties.

Serological behaviour

Rabbits were bled 7 days after being injected separately with 10 mg. of each of the forms of the cowpea virus. Both antisera precipitated with their homologous antigens, but only at dilutions up to 1/40; the antiserum to the bean form also precipitated the heterologous antigen, but the antiserum to the tobacco form did not. When bled 7 days after a third injection of 10 mg., both antisera had precipitation titres greater than 1/1000 and both now precipitated their homologous and heterologous antigens. Their precipitation behaviour with the two forms, however, differed considerably. Not only was the precipitation titre of the sera much smaller with the heterologous than with the homologous antigens, but, as Table 1 shows, in tests with decreasing antigen and constant antiserum concentrations the homologous antigens precipitated more rapidly and over a much wider range of antigen/antibody ratios than did the heterologous antigens. When either antiserum was fully absorbed by the heterologous antigen, its ability to precipitate the homologous antigen was only slightly impaired. These facts combine to show that the two forms of the virus have very few antigens in common, and to suggest differences between the two of the same order as those between type TMV and cucumber virus 3 (Bawden & Pirie, 1937), which has a known host range confined to the cucurbitaceae and which Knight (1955) thought so different from type TMV that, despite some common antigens, it should be regarded as a distinct virus.

Precipitation tests were also made with the two antisera and other strains of tobacco mosaic virus. The sann-hemp virus behaved very similarly to the cowpea virus, and although its two forms also probably have fewer than 10%

Table 1. *The precipitation of the bean and tobacco forms of the cowpea virus by their homologous and heterologous antisera*

Times are the intervals between the beginning of incubation at 50°, after mixing antigen and antibody, and the recording of the amount of precipitation.

Antiserum and dilution	Time	Bean form antigen at mg./l.					
		100	50	25	12	6	3
		Degree of precipitation					
Bean, 1/20	5 min.	+++	++	+	−	−	−
	4 hr.	++++	++++	+++	++	+	±
Bean, 1/100	5 min.	+++	++	+	−	−	−
	4 hr.	+++	+++	+++	++	++	+
Tobacco, 1/20	30 min.	−	+	+	−	−	−
	4 hr.	++	++	++	+	±	−
Tobacco, 1/100	2 hr.	−	−	−	+	−	−
	4 hr.	−	−	±	+	±	−
		Tobacco form antigen at mg./l.					
		100	50	25	12	6	3
		Degree of precipitation					
Bean, 1/20	30 min.	−	+	−	−	−	−
	4 hr.	+++	+++	+++	+++	++	+
Bean, 1/100	4 hr.	−	−	−	−	+	+
Tobacco, 1/20	5 min.	+++	++	+	−	−	−
	4 hr.	++++	++++	++++	+++	++	+
Tobacco, 1/100	5 min.	+++	++	+	−	−	−
	4 hr.	++++	++++	++++	+++	++	+

Signs (++++, +++, ++, +, ±) indicate the relative sizes of the precipitates; − = no precipitate

of their antigens in common, each is serologically similar to the comparable form of the cowpea virus. The other strains tested all behaved with the antiserum against the tobacco form in much the same way as they do with antisera to type TMV, which obviously is antigenically very similar to the tobacco forms of the cowpea and sann-hemp viruses. When tested against the antiserum to the bean form, type TMV, tomato aucuba mosaic virus and the tobacco form of the sann-hemp virus were all precipitated to much the same extent as the tobacco form of the cowpea virus, but strain U2 (Siegel & Wildman, 1954) and one that causes a systemic mosaic in *Datura tatula* were not precipitated. That these two strains probably have a very few antigens in common with the bean form, however, was suggested by the formation of small precipitates when a preparation of the bean form of the cowpea virus was titrated against their antisera; precipitates formed only when these antisera, which with their homologous antigens have titres in excess of 1/1000, were used at dilutions less than 1/25, and then they formed over only a narrow zone of antigen/antibody ratios and after prolonged incubation at 50°. No attempt was made to assess the precise degree of serological relationships between the different strains by detailed cross-absorption experiments. The results, indeed, would be of doubtful value, for the antibody contents of different sera prepared against one preparation of one strain differ qualitatively as well as quantitatively, depending on the amount of antigen injected, the number of injections and the relative extent to which the animal responds to quantitatively major and minor antigens.

Other strains of tobacco mosaic virus that have been found to differ considerably in their antigenic constitution have been found also to have considerably different amino acid compositions. Analyses made by Mr M. W. Rees of the Biochemical School, Cambridge University, show that there are also considerable differences between the amino acid compositions of the two forms of the cowpea virus. The most striking is that the bean form contains histidine, which does not occur in the tobacco form and has been previously recorded in only the rib-grass strain of TMV (Knight, 1947), but it also contains more arginine and less lysine than the tobacco form. That the histidine is not present in a normal bean protein contaminating the purified preparation of the bean form was shown by propagating this form of the virus in tobacco. This was done by inoculating tobacco plants with the bean form, keeping them at 32° continuously for 8 days, when the inoculated leaves were harvested and the virus purified. The preparation caused only small slowly developing lesions when inoculated to *Nicotiana glutinosa*, precipitated with antisera in the manner of the bean form and Mr Rees found it to contain the same amount of histidine as preparations made from systemically-infected beans.

Electrophoretic behaviour

When purified preparations of the tobacco form were examined at 0·2% in the electrophoresis apparatus they behaved much like preparations of type TMV and showed a single component with a tall slim peak of mobility about $-9{\cdot}4 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ V.$^{-1}$ (Pl. 2, fig. 5), but at 20 mg./ml. another component with mobility $-4{\cdot}7 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ V.$^{-1}$ was detectable. Preparations of the bean form at 2 mg./ml. usually showed two distinct components (Pl. 2, fig. 6), the major with a mobility of about $-4{\cdot}7$ and the minor of $-9{\cdot}4 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ V.$^{-1}$. Hence, components with the same mobilities are detectable in preparations of both forms of the virus, but the component that is minor in one becomes the major in the other.

At 20 mg./ml. there was evidence of additional components in the bean form, and as shown below, these were also once noted at lower concentrations. The peaks are less slim than with the tobacco form and broaden more as electrophoresis proceeds. This suggests that the particles are smaller, as also does the fact that the bean form precipitates with its antiserum more slowly and its floccules are more compact than those of the tobacco form. Electron micrographs further suggest this, for those of purified preparations of the bean form show most particles to be shorter than 300 mμ., unlike preparations of the tobacco form, but no attempt was made to estimate the difference by counting random particles of the two. Also, ultracentrifugation adequate to sediment preparations of the tobacco form leaves material in the supernatant fluid of purified preparations of the bean form. This material was not studied in any detail but electron micrographs of the supernatant fluid after centrifuging for 1 hr. at 50,000 *g* showed it to contain few particles as long as 100 mμ. and, weight for weight, it had only $\frac{1}{50}$th the infectivity of the material that sedimented. It absorbed ultraviolet radiation at 2600Å rather less strongly than normal preparations, suggesting that it probably contained less than 6 % nucleic acid. When examined electrophoretically at about 2 mg./ml. it gave a single peak moving with the speed of the minor component of the preparations of the bean form (Pl. 1, fig. 7). However, centrifugation did not separate the two components completely, for solutions of the re-suspended pellet contained both.

There are obvious similarities between the small particles present in the preparations of the bean form of the cowpea virus and the material that Takahashi & Ishii (1953) called X-protein, which remains in the supernatant fluid when sap from tobacco plants infected with type TMV is ultracentrifuged. However, free X-protein does not accompany TMV through purification procedures and it forms a much smaller proportion of the total anomalous protein present in sap than does the minor component of the bean-form preparations. The ratio of the minor and major components varied considerably in different preparations of the bean form of the cowpea virus, but whether this reflects variations in the amounts originally present in the sap or variable losses of the minor component during purification is unknown. Every preparation from bean was found to have at least these two components, but when the purified preparation of the bean form from inoculated tobacco plants kept at 32° was examined at 2 mg./ml., this showed only one, which had the mobility of the major component of the bean preparation. Again, it is unknown whether this was because the minor component is not formed under these conditions or whether it was lost during purification.

That the minor component in preparations of the bean form contains infective particles was shown by separating the two components electrophoretically. When inoculated to *Nicotiana glutinosa* at similar concentrations each gave similar numbers of the small slowly developing lesions. Each also produced similar systemic symptoms on French beans as well as many local lesions of the type shown in Pl. 1, fig. 1. When virus prepared from beans inoculated with either the separated major or minor component was examined at 2 mg./ml., it gave electrophoretic patterns with the usual two components. Tobacco plants inoculated with either the minor or major component all produced local lesions; not all became systemically infected and more did from inoculations with the separated minor component than from those with the major. Later inoculations from beans infected with either of the two separated components produced systemic infections in tobacco regardless of whether the component had previously infected tobacco systemically.

Serological tests with the two separated components showed no great differences between their behaviour. Each was precipitated by antiserum against the bean and tobacco forms of the cowpea virus, and each was precipitated more rapidly and over a wider range of antigen/antibody ratios by the bean-form antiserum than by the tobacco-form antiserum. Thus although the minor component has much the same electrophoretic mobility as the tobacco form of the virus, it seems to be no more similar to it antigenically than is the major component, which moves much more slowly.

The sann-hemp virus behaved in much the same way as the cowpea one. Purified preparations of the tobacco form at 2 mg./ml. behaved like type TMV and showed one component; preparations of the bean form showed a major component with mobility $-4{\cdot}2$ and a minor one with mobility $-9{\cdot}7 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ V.$^{-1}$. When these two components were separated electrophoretically and inoculated to *Nicotiana glutinosa*, each produced only small slowly developing local lesions. Each infected beans systemically, produced local lesions in tobacco and infected some plants systemically. All purified virus preparations from beans infected systemically by inoculation with either component again showed the two usual components when examined electrophoretically, and the preparation from one batch of beans inoculated with a separated minor component showed two additional components with approximate mobilities of -7 and -5×10^{-5} cm.^{2} sec.$^{-1}$ V.$^{-1}$ (Pl. 2, fig. 8).

Inactivation by ultraviolet radiation

Table 2 shows the results of infection tests with unirradiated preparations of the bean and tobacco forms of the cowpea virus and with samples of the same preparations after exposure for different lengths of time to ultraviolet (u.v.) radiation. The source of the radiation was a Vitreosil low-pressure mercury lamp, most of whose radiation is of wavelength 2537 Å. Solutions containing 200 mg. purified virus preparation/l. were irradiated as layers 0·14 cm. deep in Petri dishes placed 20 cm. from the lamp, where the intensity of the radiation was 870 μW./cm.^{2}. The dishes were rocked continually during the period

Table 2. *The inactivation of the bean and tobacco forms of the cowpea virus by ultraviolet radiation*

Preparations at 200 mg./l. were irradiated as described in the text and diluted to the stated concentrations before inoculation to *Nicotiana glutinosa*.

Treatment	Inoculum concentration (mg./l.)	Mean no. lesions/ half leaf
	Bean form	
Unirradiated	10	36
Unirradiated	3·3	25
Unirradiated	1·0	8
Irradiated, 40 sec.	20	4·5
Irradiated, 80 sec.	66	3·5
Irradiated, 120 sec.	200	2·3
	Tobacco form	
Unirradiated	10	98
Unirradiated	3·3	43
Unirradiated	1·0	30
Irradiated, 80 sec.	20	65
Irradiated, 160 sec.	66	26
Irradiated, 240 sec.	200	13

of exposure, a treatment that is assumed to have ensured that all particles were equally exposed. The control and irradiated preparations were then inoculated at various dilutions to *Nicotiana glutinosa*. The tobacco form is obviously much more resistant to inactivation than the bean form; after exposure for 160 sec. it still had about $\frac{1}{60}$th of its initial infectivity, whereas the bean form retained only about $\frac{1}{40}$th after 40 sec. and less than $\frac{1}{500}$th after exposure for 120 sec. Siegel & Wildman (1954) found that different strains of tobacco mosaic virus differed in their sensitivity to inactivation by u.v. radiation and the differences between the two forms of the cowpea virus seem to be of the same order as those between the resistant type TMV and their most sensitive strain U2. Strain U2, which was derived from a stock of type TMV, also has the same electrophoretic mobility as the major component in bean preparations of the cowpea and sann-hemp viruses, but it seems to be genetically more stable than the cowpea virus, for Siegel & Wildman (1954) stated that it 'never reverted to common TMV in spite of numerous transfers over a 3-year period'. It also gives smaller local lesions in *N. glutinosa* than does type TMV, but these appear at the same rate; I could get no infections in French beans with strain U2, which despite some similarities with the bean form of the cowpea virus also seems to have fewer antigens in common with it than does type TMV.

The change from bean to tobacco form

When the tobacco form of the cowpea virus is mixed in various amounts with a constant amount of the bean form and the mixtures inoculated to *Nicotiana glutinosa*, the lesions produced by the tobacco form appear in the usual time of 2 days and they remain readily distinguishable from those produced later by the bean form. As the number of lesions produced by the tobacco form also seems little affected by the presence of the bean form, inoculations to *N. glutinosa* provide a convenient method of detecting when the tobacco form first appears in leaves inoculated with the bean form. This change-over was studied by inoculating leaves with the bean form, washing them thoroughly to get rid of virus on the leaf surface, and then macerating samples from them at intervals for inoculation to *N. glutinosa*.

When tobacco leaves are inoculated with the tobacco form of the cowpea virus less virus is usually recovered in extracts 24 hr. after inoculation than 1 hr. afterwards, but after 48 hr. the amount has usually increased considerably; after 3 days there is usually detectable multiplication, even when the original inoculum was highly diluted, and from then on the amount increases rapidly. When similar leaves are inoculated with undiluted infective bean sap, or better (because bean sap contains substances that interfere with infections in tobacco and *Nicotiana glutinosa*) with 1 mg./ml. of purified bean-form virus, extracts made 24 or 48 hr. after inoculation usually contain less virus than extracts made 1 hr. after inoculation. Newly produced virus begins to be detectable about 3–4 days after inoculation, when the leaves start to show lesions, but the number of lesions produced on *N. glutinosa* by extracts taken at this time are few compared with those from plants inoculated with the tobacco form. All the lesions at this time are of the small slowly developing type, and the number of such lesions does not increase greatly with increase in the period of time at 20° between inoculation of the original tobacco leaves and the preparation of the extracts. From many inoculated leaves, this is the only result of such return inoculations to *N. glutinosa*, but extracts from leaves of plants that will later develop systemic mosaic symptoms sooner or later give, in addition, the large rapidly developing lesions which are characteristic of infection by the tobacco form of the virus (Pl. 2, fig. 9). These large lesions rarely occur from inoculations with extracts made sooner than 6 days after the tobacco leaves were inoculated and sometimes not until twice as long. Extracts made at intervals after the first appearance of the large lesions usually produce large lesions in rapidly increasing numbers, but not always so, presumably because sometimes the first sampling of the tobacco leaves has removed the only site or sites where the tobacco form of the virus was multiplying.

No exhaustive search has been made to find the range of plants in which the change from bean to tobacco form occurs, but it is not restricted to tobacco. It occurs in other species of *Nicotiana*, such as *N. bigelovii* and *N. clevelandii*, but has not been noticed in *N. glutinosa*; it also occurs in other solanaceous plants, such as *Datura tatula* and the tomato, and in at least one plant from the *Chenopodiaceae*, namely sugar beet. From leaves of these species inoculated with preparations of the bean form, which themselves gave no large rapidly developing lesions in *N. glutinosa*, extracts made during the first few days after inoculation also produced only lesions characteristic of infection with the bean form, whereas extracts made after a week or more produced lesions of both types.

Studies of the course of infection when bean leaves are inoculated with the tobacco form of the cowpea virus are complicated by the small virus content of such leaves and the presence of substances that inhibit infections in *Nicotiana glutinosa*. However, such tests as were made showed that virus obtained in extracts made within 5 days of inoculation produced only lesions characteristic of infection by the tobacco form, whereas later ones often produced a few lesions characteristic of infection by the bean form.

DISCUSSION

The main point that calls for discussion is the mechanism whereby the cowpea and sann-hemp viruses change their constitution and properties when they are transferred between solanaceous and leguminous hosts. The novelty about these viruses is the magnitude of the changes they undergo and that the changes are reversible, for there is nothing new in the discovery that strains of TMV vary or that the behaviour of cultures alters when they are passed through a new host. Most variations that have previously been described, however, have been changes in virulence, although strain U2 which seems to have derived from type TMV also differs from it in several other ways (Siegel & Wildman, 1954). Also, changes resulting from passage through a new host plant have previously been explicable by inocula containing more than one strain, not all of which invade the new host systemically. Thus Johnson (1947), who found that bulk cultures of TMV were always less virulent towards tobacco after passage through sea-holly (*Eryngium aquaticum* L.), readily explained this by experiments with single-lesion isolates, which showed that bulk cultures contained both virulent and avirulent forms and that the virulent ones gave only local lesions in sea-holly. Single-lesion isolates of the cowpea virus, however, changed as readily as did bulk cultures, and the reversibility of the change also seems inexplicable by postulating that different hosts act solely by differentially favouring one strain out of several introduced in the inoculum.

It cannot safely be assumed that individual lesions in *Nicotiana glutinosa* are caused by single virus particles, but what evidence there is suggests that it is rare for widely separated lesions, produced by dilute inocula in which two strains are mixed, to contain both strains (Kunkel, 1934; Lauffer & Price, 1945). Although the 'purity' of any culture is always suspect, it seems improbable that the tobacco form of the cowpea virus is carried as such through local lesions produced by inoculations with the bean form. Not only does the tobacco form produce its characteristic lesions when it is added in small amounts to large quantities of the bean form, but it produces them in approximately the same numbers as when inoculated on its own. Also, even if its presence were not immediately obvious from the character of the lesion, it should become so after a second passage in *N. glutinosa*, for extracts from lesions caused by the tobacco form are much more infective than those from the bean form. However, when the single-lesion isolates were passed successively through *N. glutinosa*, they gave no sign of containing the tobacco form.

Similarly, the results of inoculating Pinto beans give no reason to suggest that sap from systemically-infected tobacco contains both forms of the virus in transmissible amounts. This variety gives only black local lesions when inoculated with the tobacco form of the virus and no such local lesions but a systemic mosaic disease when inoculated with the bean form. Inoculations from such mottled leaves can cause systemic infections in tobacco, but the virus that becomes systemic gives only black local lesions when returned to Pinto. When inoculated to Prince instead of Pinto beans, it can lead to a systemic infection, and the virus from Prince will then readily infect Pinto systemically and cause no black local lesions. Thus, starting with virus that is causing a systemic mosaic disease in Pinto, the ability to do this is lost by passage through tobacco and regained by passage from tobacco through Prince.

None of these results seems compatible with the idea that hosts act as one-way filters, which allow only one of the types that initially infect to pass the inoculated leaves. Nor does the fact that inoculating tobacco plants with the bean form of virus leads to the production of the tobacco form in young plants at 20°, but not at 30° or in older plants, for when present the tobacco form multiplies readily in all three types of plant. Such facts and many others all combine to suggest that the tobacco form occurs in tobacco plants inoculated with the bean form, not because of its presence in the inoculum, but only after the bean form has multiplied there and because of this multiplication.

The change from either form of the virus to the other seems explicable only by the hosts selecting variants that originate in them. An attempt was made (Bawden, 1956) to explain the differences between the two forms as phenotypic variations, with the genetical material of the virus remaining constant but combining with different proteins in solanaceous and leguminous hosts. This now seems improbable, for since Gierer & Schramm (1956) showed that nucleic acid of TMV can alone cause infections, the idea has become to some extent testable. When preparations of the cowpea virus in its two forms were disrupted with phenol to produce infective preparations of nucleic acid, each form still produced its characteristic type of local lesion in *Nicotiana glutinosa*.

The only objection to the idea that the two forms can change into one another by events equivalent to reversible mutations seems to rest on the apparent suddenness with which such large changes occur. The differences between the two forms are perhaps greater than those between any two previously recognized strains of TMV, and most other strains that differ considerably can reasonably be assumed to have reached their current states by changes accumulated over long periods of evolution along separate paths. However, differences in the size and rate of change seems no adequate reason for postulating a different mechanism. In terms of population statistics, there is no reason to assume that the change happens so very often or that it happens as a 'single-step' mutation. With the best conditions for the change from the bean to the tobacco form, not every tobacco plant becomes systemically infected, even when 3–4 leaves are dusted with 'Celite' and rubbed over their whole surfaces with inoculum of the bean form at 1 mg./ml. Such a treatment may give hundreds of local infections per leaf and each lesion will contain many millions of virus particles, produced by multiplication cycles in a succession of infected cells. Possibly only one particle of the tobacco form needs to occur in such conditions to lead to a systemic infection, so the mutation rate need not be fantastically high. Furthermore, if the many differences in behaviour between the two forms reflect many genetical differences, not all the required mutations need necessarily have occurred in one particle; in the course of multiplication, particles in which different mutations occur may be able to exchange genetic material.

The multiplication of type TMV does not lead to a uniform product all exactly resembling the infecting particles, but to a variety of products differing in constitution, size and infectivity (Bawden & Pirie, 1945, 1956). That there is even a greater variability in the products when the cowpea virus multiplies, especially in beans, is evident from the electrophoretic patterns. Infectivity tests give no evidence that the variability extends to the occurrence in sap from beans of particles with the infective characters of the tobacco form, for the typical lesions in *Nicotiana glutinosa* were not formed even when inoculations were made with the component having the same electrophoretic mobility as the tobacco form. However, this failure does not mean that no such particles occur. To get infections by rubbing leaves requires the use of inocula containing at least 10^7 particles/ml., so that many could lie hidden in sap from beans. Hence, when multiplying in any host each form of the virus may occasionally produce particles of the other form, but only when the occasional particles are produced in hosts which they, but not their parents, readily invade systemically can they multiply and reach concentrations at which their presence becomes detectable. The only experimental evidence that seems difficult to reconcile with this idea of undirected and random mutations leading from one form to another, is the failure of large tobacco plants to become systemically infected by inocula of the bean form that give systemic infections in young seedlings, for the tobacco form seems to multiply and move with almost equal readiness in plants of both ages. One explanation might be that the bean form itself multiplies much less in the larger plants so that, with a constant mutation rate, there would be fewer opportunities for the tobacco form to be produced. However, the possibility of the species and physiological condition of the host plant playing some part in producing mutations, in addition to selecting those forms best able to invade it, cannot be excluded.

That variants do occur in single-lesion isolates of the tobacco form when multiplying in tobacco is easily demonstrable by selecting inoculum from the occasional yellow spots that occur. When this was used to infect further tobacco plants, it produced a general yellow mosaic; inoculations from such plants infected Prince beans systemically, but produced the same kind of symptoms as did unselected inoculum. Also, when returned to tobacco, the systemic symptoms there were again of the usual type; similarly, when continually passaged in tobacco without selecting inoculum from the yellow areas, the symptoms reverted to the original type. Hence, it seems that the reversible changes can be of more than one type, some simply affecting virulence towards tobacco without greatly altering the physico-chemical properties, and others leading to much larger differences.

In addition to those mentioned in the text, I am also greatly indebted to Dr A. Kleczkowski for making all the electrophoretic measurements and separations and to Mr H. L. Nixon for making electron micrographs.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. A first-formed leaf of French bean (*Phaseolus vulgaris*) var. Prince, photographed 10 days after it was inoculated with the bean form of the cowpea virus, when it showed many yellow and white rings and spots.

Fig. 2. Deformed and mottled leaves from French bean, var. Prince, photographed 5 weeks after the plant was infected with the cowpea virus.

Fig. 3. Leaf of *Nicotiana glutinosa* showing the kinds of lesions produced by the bean and tobacco forms of the cowpea virus in plants kept under the usual glasshouse conditions. Photographed 1 week after inoculation: left-hand half of leaf shows the small white lesions produced by the bean form and the right-hand the larger lesions produced by the tobacco form.

Fig. 4. Leaf of *Nicotiana glutinosa* showing the large chlorotic rings (left-hand half) produced by the tobacco form of the cowpea virus in plants kept continuously at 36°; the right-hand half of the leaf is infected with the bean form, which multiplies at this temperature but causes no lesions. Photographed 1 week after leaf was inoculated.

Plate 2

Fig. 5. Electrophoretic pattern of a purified preparation of the tobacco form of the cowpea virus, showing a single sharp peak with a mobility of $-9{\cdot}4 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ V.$^{-1}$.

Fig. 6. Electrophoretic pattern of a purified preparation of the bean form of the cowpea virus showing two peaks, of which the larger has a mobility of $-4{\cdot}7$ and the smaller of $-9{\cdot}4 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ V.$^{-1}$.

Fig. 7. Electrophoretic pattern of a preparation, made from the unsedimented material after a purified preparation of the bean form of the cowpea virus was ultracentrifuged at 50,000 *g*, showing a single peak with a mobility of $-9{\cdot}4 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ V.$^{-1}$.

Fig. 8. Electrophoretic pattern of a purified preparation of the bean form of the sann-hemp virus, showing four components with mobilities of $-9{\cdot}4$, -7, -5 and $-4{\cdot}7 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ V^{-1}. The French beans from which this preparation was made were infected by inoculation with the slower component, separated electrophoretically from a preparation that contained only the usual two.

Fig. 9. Leaf of *Nicotiana glutinosa* showing lesions produced by inoculation with extracts made from tobacco leaves one week after they were inoculated with the bean form of the cowpea virus. Right-hand half inoculated with 'Celite' to increase numbers of lesions. Most of the lesions are typical of those produced by the bean form, and this was the only type given by earlier extracts; but now there are also some larger and more distinctive lesions, typical of those produced by the tobacco form.

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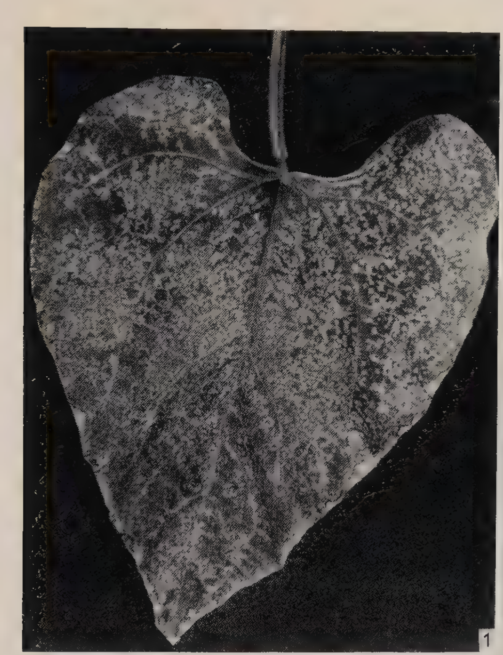

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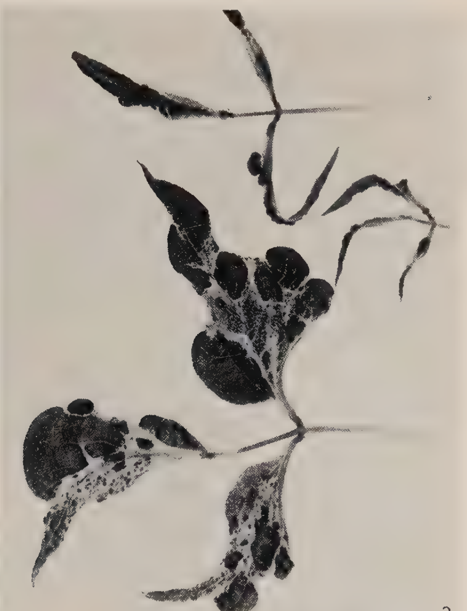

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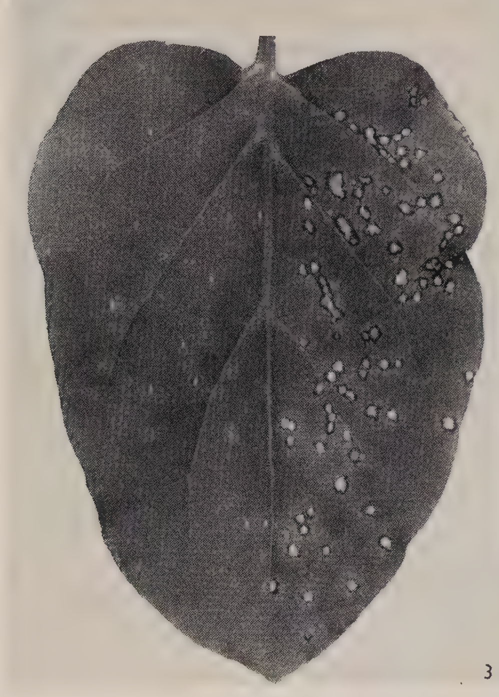

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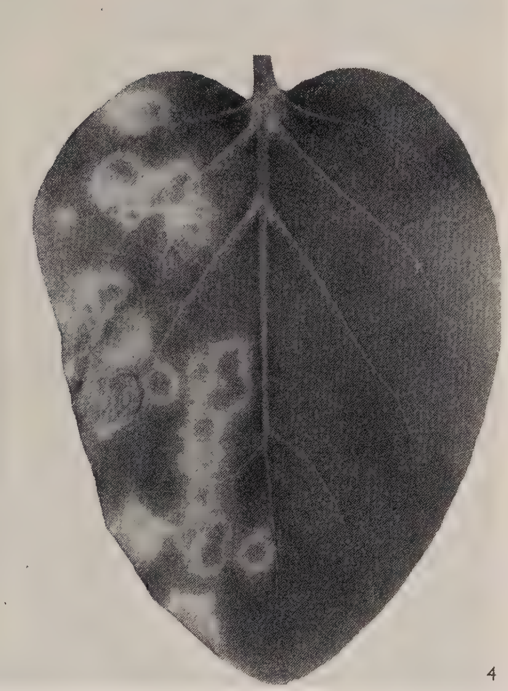

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F. C. Bawden—Reversible changes in tobacco mosaic viruses. Plate 1

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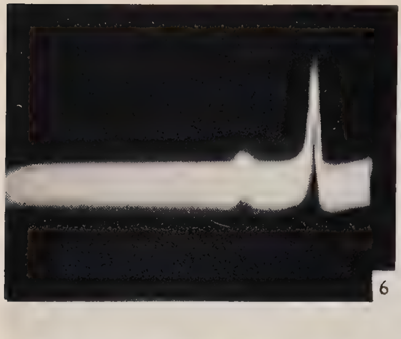
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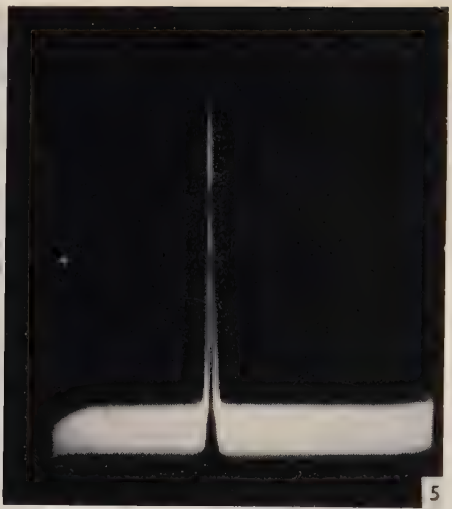
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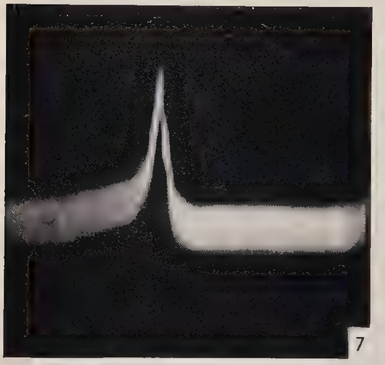
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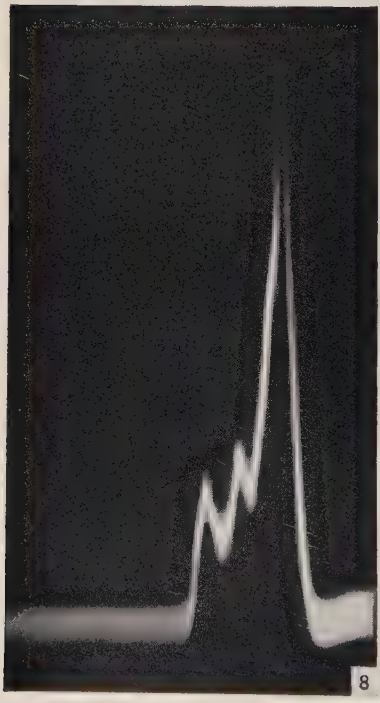
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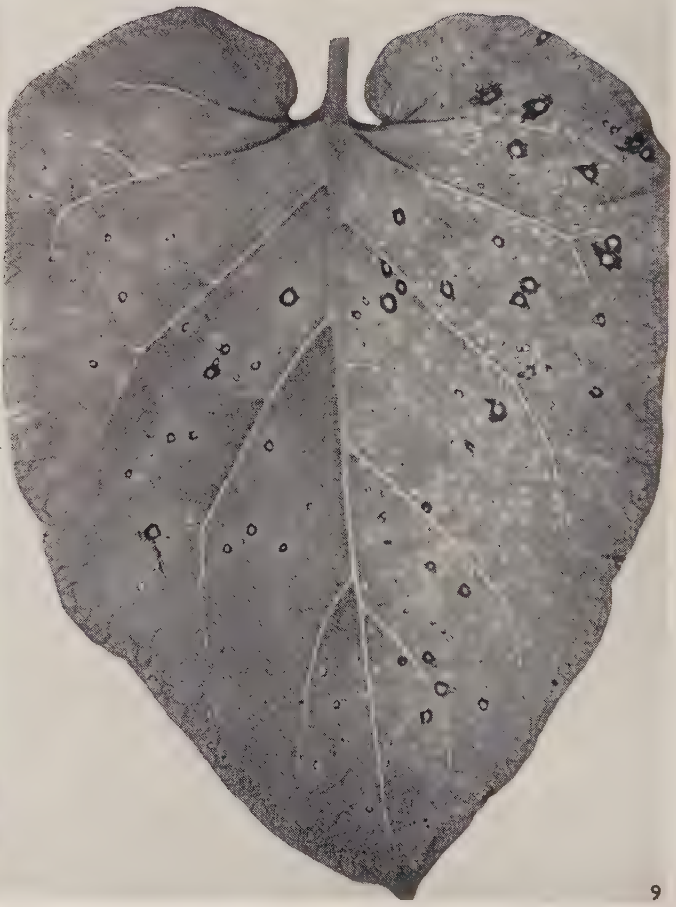
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F. C. BAWDEN—REVERSIBLE CHANGES IN TOBACCO MOSAIC VIRUSES. PLATE 2

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The Action of Chloramphenicol on Protein and Nucleic Acid Synthesis by *Escherichia coli* Strain B

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SUMMARY: Growth-inhibitory concentrations of chloramphenicol caused a prompt inhibition of protein synthesis in *Escherichia coli* strain B. This inhibition was accompanied by alteration in nucleic acid synthesis from a logarithmic to a linear rate, and by the excretion into the medium of free purine and pyrimidine bases, three of which were identified as guanine, hypoxanthine and uracil. At slightly lower concentrations the antibiotic caused a slight stimulation of nucleic acid synthesis.

Gale & Folkes (1953*b*) showed that chloramphenicol at bactericidal concentrations exerted a specific inhibition of protein synthesis by washed suspensions of *Staphylococcus aureus*. Such concentrations stimulated nucleic acid synthesis and were without effect on glucose fermentation. Wisseman, Smadel, Hahn & Hopps (1954) with *Escherichia coli* showed that the antibiotic promptly inhibited protein synthesis while allowing nucleic acid synthesis to continue 'temporarily unabated'. Ammonia assimilation was also largely suppressed, but continued at a rate compatible with nucleic acid formation. The present paper describes the results of related studies carried out with *E. coli* strain B.

METHODS

Organism and growth conditions. The organism used throughout this work was *Escherichia coli* strain B. It was maintained by daily subculture in a glucose + inorganic salts medium (Abelson, Bolton & Aldous, 1952), modified by increasing the content of Na_2HPO_4 to 9·0 g./l., and of KH_2PO_4 to 4·5 g./l.; glucose was present at a concentration of 0·5 % (w/v). Organisms grown in the above medium for 15 hr. at 37° in Roux bottles were harvested on the centrifuge, washed twice in 0·85 % (w/v) NaCl solution and suspended in saline to a concentration equivalent to 45 mg. dry wt. organism/ml. Dry weights of suspensions of organisms were estimated turbidimetrically by using an Hilger adsorptiometer and a previously prepared calibration curve of optical density against equivalent dry weight of suspensions of the organism.

Growth-inhibition coefficients. The minimum growth inhibitory concentration of chloramphenicol was determined by inoculating with *c.* 10^6 organisms/5·0 ml., tubes containing serial dilutions of chloramphenicol in growth medium. These tubes were incubated at 37° for 48 hr. Using turbidity to the naked eye as the criterion of growth, it was found that growth of the organism was inhibited by 3·0 μg. chloramphenicol/ml.

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Synthesis of protein and nucleic acid. Optimum synthesis of protein and nucleic acid occurred when organisms prepared as above were incubated at a concentration equivalent to 1·5 mg. dry weight organism/ml. in growth medium at 37° with vigorous aeration. The synthesis of these components which occurred in the presence of different concentrations of chloramphenicol was compared with that observed in a control without chloramphenicol.

Estimation of protein. Organisms were centrifuged from 5·0 ml. portions of the incubation mixtures, washed twice in saline and resuspended in water. The protein was precipitated by the addition of an equal volume of cold 10% (w/v) trichloroacetic acid (TCA). The mixture was stored at 0° for 1 hr., the precipitate separated and washed with cold 5% (w/v) trichloroacetic acid. The protein was hydrolysed with 6 N-HCl for 15 hr. at 105° in sealed tubes. Excess hydrochloric acid was removed *in vacuo* and the residue suspended in 0·2 M-sodium acetate buffer (pH 4·5). The glutamic acid of the hydrolysate was determined by using the specific decarboxylase method of Gale (1945). This value for 'combined glutamate' was taken as a measure of the protein content of the organisms (Gale & Folkes, 1953*a*).

Estimation of nucleic acid. The nucleic acid content of the organisms was determined by using the trichloroacetic acid-precipitable fraction of whole organisms prepared as above. After washing with cold 5% (w/v) trichloroacetic acid, the residue was extracted with three successive 2·0 ml. portions of 5·0 (w/v) trichloroacetic acid at 90° for 10 min. The optical density at 260 mμ. of suitable dilutions of the combined extracts was determined against a trichloroacetic acid blank similarly treated. The nucleic acid content of the organisms was calculated by assuming an extinction coefficient of 24 for a 0·1% solution of nucleic acid (Gale & Folkes, 1954). All ultraviolet absorption determinations were carried out with a Beckman spectrophotometer model DU.

Analysis of the medium. The incubation medium, after removal of the organisms fluoresced under u.v. light and absorbed strongly in the region 240–260 mμ. On repeated freeze-drying, after addition of excess NaOH to remove ammonia, the residue gave a positive ninhydrin reaction. The fluorescent and u.v.-absorbing constituents of the medium were removed from solution by adsorption on to activated charcoal (pre-treated as described by Partridge & Brimley, 1952). The adsorbed components were eluted with 5% (w/v) phenol in 20% (v/v) acetic acid. The phenol was extracted with ether and the acetic acid removed by evaporation to dryness *in vacuo*. The residue was dissolved in the minimum amount of distilled water. Single dimensional chromatograms were prepared and developed with the following solvents: *n*-butanol + formic acid + water (77:10:13), *n*-propanol + 0·02 M-potassium acetate (pH 6·0) (70:30), isopropanol + water (65:35) containing 2 N-HCl. The dried papers were examined under u.v. radiation and the areas of fluorescence outlined. The positions of the u.v.-absorbing components were determined by preparing direct contact prints on Ilford Document Paper, using a mercury discharge lamp (Thermal Syndicate Ltd., T/M5/369) as u.v. source. The corresponding areas on the chromatograms were cut out and eluted with 0·1 N-HCl and the adsorption spectra of the compounds determined against similar eluates of equivalent blank strips of paper cut from the chromatograms. Duplicate papers run in the same solvents were dryed and sprayed with 0·1% (w/v) ninhydrin in chloroform and heated at 100° for 5 min.

RESULTS

When suspensions of *Escherichia coli* strain B were incubated as described above growth and the synthesis of protein and nucleic acid proceeded at a logarithmic rate with a mean generation time of 70–90 min. Progress curves for the inhibition of protein and nucleic acid synthesis by different concentrations of chloroamphenicol were determined. The synthesis of protein is very sensitive to chloramphenicol, being completely inhibited by 3·0 μg. chloramphenicol/ml. (Fig. 1). The corresponding results for the inhibition of nucleic acid synthesis (Fig. 2) indicated that low concentrations of chloramphenicol altered the rate of synthesis from a logarithmic to a linear rate, and that increasing the concentration of chloramphenicol merely decreased the time before the onset of this alteration.

Wisseman *et al.* (1954) reported that chloramphenicol promptly inhibited protein synthesis in *Escherichia coli*, whereas the synthesis of nucleic acid was unaffected even at concentrations much greater than that which inhibited

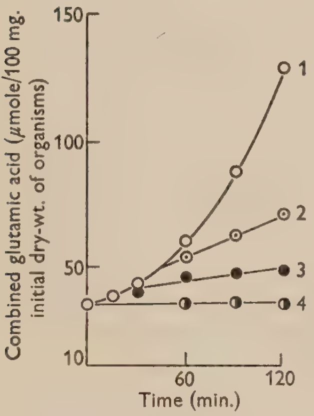

Fig. 1

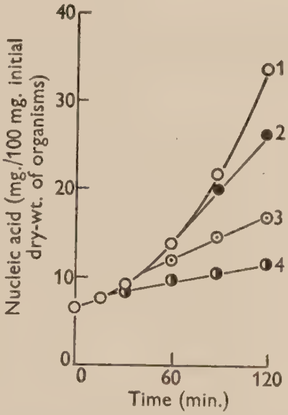

Fig. 2

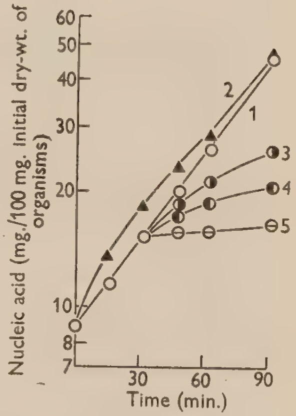

Fig. 3

Fig. 1. Progress curves for the response of protein synthesis in *Escherichia coli* strain B to different concentrations of chloramphenicol: curve 1, ○—○, 0 to 0·1 μg./ml.; curve 2, ⊙—⊙, 0·3 μg./ml.; curve 3, ●—●, 1·0 μg./ml.; curve 4, ◑—◑, 3·0 μg./ml.

Fig. 2. Progress curves for the response of nucleic acid synthesis in *Escherichia coli* strain B to different concentrations of chloramphenicol: curve 1, ○—○, 0 to 0·3 μg./ml.; curve 2, ●—●, 1·0 μg./ml.; curve 3, ⊙—⊙, 3·0 μg./ml.; curve 4, ◑—◑, 10·0 μg./ml.

Fig. 3. Progress curves for the response to different concentrations of chloramphenicol, of nucleic acid synthesis in *Escherichia coli* strain B which had been preincubated in growth medium until logarithmic growth was established. Curve 1, ○—○, shows the rate of synthesis in control without chloramphenicol; for the other curves the incubation mixtures contained chloramphenicol: curve 2, ▲—▲, 1·0 μg./ml.; curve 3, ◑—◑, 3·0 μg./ml.; curve 4, ◐—◐, 10·0 μg./ml.; curve 5, ⊖—⊖, 30·0 μg./ml.

growth. In view of the apparent discrepancy between the results of these workers and those reported here, an experiment was carried out under conditions similar to those described by Wisseman *et al.* (1954). This involved the incubation of the organisms, after harvesting, in medium without chloramphenicol 'until the logarithmic rate of growth was well established' (90 min.). Chloramphenicol was then added and the effects studied over a period of 90 min. As before, it was found that protein synthesis was completely inhibited by concentrations of chloramphenicol greater than 3·0 μg./ml. The observed effects on the synthesis of nucleic acid (Fig. 3) show that, as reported by Wisseman *et al.* (1954), growth inhibitory and higher concentrations of chloramphenicol exerted little action on the process during the first 30 min. Thereafter, however, the response was governed by the concentration of the drug, and this effect may have represented a direct inhibition of the process which was exhibited only after an initial lag of 30 min.

Gale & Folkes (1953*b*) showed that in suspensions of *Staphylococcus aureus* concentrations of chloramphenicol which completely inhibited protein synthesis caused a marked stimulation of nucleic acid synthesis. Wisseman *et al.* (1954) did not observe a similar effect with *Escherichia coli*. In the present work a slight but reproducible increase in nucleic acid production occurred with 1·0 μg. chloramphenicol/ml. (Fig. 3, curve 3). Under these conditions protein synthesis was considerably decreased (Fig. 1, curve 2). This concentration of chloramphenicol also caused a considerable increase in the u.v. absorption of the medium as compared with that in the control suspension. In both cases this increased absorption continued for at least 120 min., and samples of the media were therefore taken for analysis after this interval. The absorption spectra of 1/2 aqueous dilutions of the samples, as well as those of undiluted samples after treatment with activated charcoal, are shown in Fig. 4. Eluates of the charcoal were prepared and diluted so that comparable spectra were obtained (Fig. 5). Both sets of results indicated the presence of '260 mμ.-absorbing material', possibly free or combined purine or pyrimidine derivatives in the medium from the chloramphenicol-treated suspension.

Two dimensional chromatograms of the concentrated eluates revealed several ninhydrin-reacting substances, three of which were identified on the basis of their R_F values as valine, leucine and methionine. As there was no apparent difference between the samples with and without chloramphenicol, further analysis was not undertaken. Ultraviolet photographs of single-dimensional chromatograms showed at least four substances which absorbed radiation in the region of 260 mμ. in the chloramphenicol-treated suspension. On the basis of R_F values (Table 1), and by applying the principle that the logarithmic plot of the extinction of a compound at concentrations which obey De Beer's Law is independent of concentration, the presence of three bases guanine, hypoxanthine and uracil was shown (Harrington, 1954). The quantitative aspect of the appearance of these free bases in the supernatant medium has not been examined.

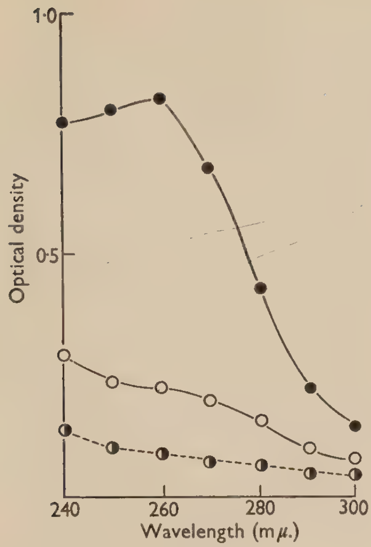

Fig. 4

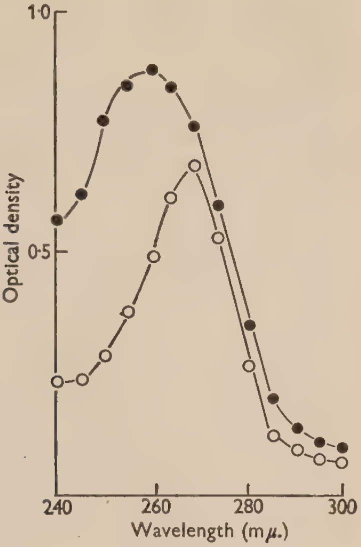

Fig. 5

Fig. 4. Absorption spectra of 1/2 aqueous dilutions of the supernatant fluid from incubations of *Escherichia coli* strain B in the absence (○—○) and in the presence (●—●) of 1·0 μg. chloramphenicol/ml. The broken line (◑-----◑) shows spectrum of undiluted samples after treatment with charcoal.

Fig. 5. Absorption spectra of 'corresponding dilutions' of the charcoal eluates prepared from the supernatant fluids from control (○—○) and chloramphenicol-containing incubations of *Escherichia coli* strain B (●—●). Dilutions were made to give spectra with closely similar extinctions.

Table 1. *Identification of u.v.-absorbing compounds*

R_F values of the 'unknown' bases excreted into the medium by chloramphenicol-treated *Escherichia coli* strain B (column A); values for the pure bases reported by Markham & Smith (1949; column B); solvent N-butanol + formic acid + water.

	A	B
Guanine	0·12–0·15	0·13
Hypoxanthine	0·30–0·35	0·30
Uracil	0·40–0·42	0·39

DISCUSSION

Gale & Folkes (1953*b*) showed that in *Staphylococcus aureus* chloramphenicol exerted a specific inhibition of protein synthesis. The results presented here (Figs. 1, 2) indicate that in *Escherichia coli* strain B there is a close parallelism between the sensitivities of protein and nucleic acid synthesis. While this difference in the behaviour of the two organisms may represent a fundamental distinction between the Gram-positive and Gram-negative species, it seems more likely that it arose from the differences in the experimental conditions. The studies on *Staphylococcus aureus* were carried out under conditions which allowed the analysis of the two relevant synthetic reactions uncomplicated by other processes of growth. In the present system both reactions were occurring as part of normal growth, and consequently may have been interdependent. This latter interpretation is clearly supported by the progress curves for the synthesis of nucleic acid. The continued formation of nucleic acid at a logarithmic rate may require the simultaneous continued production of the necessary enzymes, which presumably are protein in nature. Thus any specific inhibition of protein synthesis, in the absence of an effect on the activity of existing enzyme protein, might be expected to produce a result similar to that reported in Fig. 2. That this effect was not observed by Wisseman *et al.* (1954) under very similar conditions may have arisen from variations in the initial nucleic acid content, or in the ratio of protein to nucleic acid content of the organisms.

Britten (1954) showed that during rapid growth *Escherichia coli* strain B excreted into the medium a complex mixture of metabolic products, including amino acids, but in which carbon dioxide and acetate predominated. Dunlop (1949) reported that under certain conditions some amino acids, notably valine, leucine and methionine, might occur in detectable amounts; similar findings have been reported here. Certain concentrations of chloramphenicol caused the appearance in the medium of free purine and pyrimidine bases. Since under these conditions nucleic acid was being synthesized it seemed unlikely that these bases arose from a depolymerization of cell nucleic acid. In fact with slightly lower concentrations of chloramphenicol a stimulation of nucleic acid production was observed. It is possible that for *Escherichia coli* strain B the combined effects of chloramphenicol on nucleic acid synthesis and on the excretion of free bases may be the manifestation of an effect corresponding to the stimulation of nucleic acid synthesis in *Staphylococcus aureus* reported by Gale & Folkes (1953*b*). It seems more likely, however, that the observed effects are the results of a series of reactions consequent on the inhibition of protein synthesis by chloramphenicol. Thus, should nucleic acid polymerization require the presence of a protein template (Haurowitz, 1950) the cessation of protein synthesis might lead to saturation of the existing template. The continued production of the purine and pyrimidine bases, a process insensitive to the drug, would then be followed by the excretion of excess bases into the medium.

I wish to express my sincere thanks to Dr E. F. Gale, F.R.S., for his interest and advice throughout this work and to Dr K. McQuillen and Dr R. Davies for many helpful discussions. The work was carried out during the tenure of the Travelling Studentship in Biochemistry (1952) from the National University of Ireland.

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'*Streptococcus bombycis*', its Taxonomy and Pathogenicity for Silkworm Caterpillars

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SUMMARY: It was found that the streptococci known as *Streptococcus bombycis* have affinities with *Streptococcus faecalis* and *S. faecium*. *S. bombycis* is not a well-defined species and its entity is doubtful; it is proposed, therefore, that the name *S. bombycis* and its synonyms, which are given, be rejected. These streptococci are pathogenic for the silkworm, causing a disease of the gut. The mechanism of the pathogenicity is discussed.

The streptococcal diseases of the silkworm, *Bombyx mori* L., have been known for a long time, but neither their origin nor their aetiology has been satisfactorily explained. In the past '*Streptococcus bombycis*' was designated as a cause of '*gattine*' (*schindsucht, macilenza*), but later this disease was found to be of virus origin, and streptococci were only secondary invaders. A summary of these views was given by Steinhaus (1949). A precise determination of *S. bombycis* has not been made and the taxonomy of the organism is doubtful. It is not known who first used the name, which is thus connected with various authors. Pasteur when investigating 'ferment au chapeletes de grains', considered the bacterium to cause 'flacherie' and from this investigation the designation *Streptococcus bombycis* (Pasteur) Flügge 1886 has its origin. *Streptococcus bombycis* Zopf 1884 and *Streptococcus bombycis* Sartirana & Paccanaro 1906 have also been described. The synonymy is confused and not fully known: *Bergey's Manual* (1948) quotes (p. 254) *Streptococcus bombycis* as one of the synonyms for *Micrococcus bombycis* (Naegeli) Cohn, but a protozoon, *Nosema bombycis* Naegeli, is meant. In another place (p. 337) in *Bergey's Manual* it is stated that *Streptococcus bombycis* Sartirana & Paccanaro is not a synonym for *Streptococcus bombycis* Zopf. Paillot (1928) considered *Streptococcus bombycis* Sartirana & Paccanaro and *Streptococcus pastorianus* Krassilstschik 1896 to be identical; but this view was not shared by Krassilnikov (1949). In addition, the name *Diplococcus bombycis* Paillot, 1922, may be found in the literature, but some workers consider it also to be a streptococcus (Steinhaus, 1949). From this review it is clear that taxonomically and nomenclaturally the position of organisms named *Streptococcus bombycis* is doubtful. The difficulty is that the original description and the original cultures are lost and the descriptions which are available contain only general features so that a correct determination is impossible. All descriptions of *Streptococcus bombycis* agree that it is Gram-positive, and the cocci, 0·7–0·9 μ. in size, are arranged in short or long chains. The micro-organism grows well on ordinary solid media and forms small colonies; gelatin is not liquefied and litmus milk is acidified. The recent description by Masera (1954) does not add any useful information. Seelemann (1942) was the first to try to determine strains of *S. bombycis* by methods used for streptococci and stated that *S. bombycis* belonged to the group of enterococci with features similar to *S. faecium*. Seeleman's taxonomy of enterococci differs from more recent schemes of this group of bacteria; in his manual of streptococci (Seelemann, 1948) we find *S. faecium* as one species and *S. glycerinaceus*, as a synonym of *S. faecalis*, as another. In recent taxonomic studies of enterococci (Shattock, 1955; Lake, Deibel & Niven, 1957) a number of differences between *S. faecalis* and *S. faecium* are given, but these do not invalidate Seelemann's determination. Seelemann was the first to determine *S. bombycis* with approximate accuracy, a fact to be recognized by all workers in insect pathology. In this paper I discuss the taxonomic position of organisms named *Streptococcus bombycis*, using biochemical features and infection experiments on silkworms.

METHODS

Strains of streptococci were isolated from epizootics in populations of silkworms; the characters of these strains resembled those described for *Streptococcus bombycis*. To my strains were added strains isolated from healthy silkworms, and, for comparison, one strain each of *S. faecalis* and *S. faecium*. The designations of strains and their sources are given in Table 1. The methods used were those given in Seelemann's monograph (1948) and in papers by Shattock & Hirsch (1947), Skadhauge (1956), Shattock (1955), Barnes (1956)

Table 1. *Source and designation of strains of streptococci examined*

Designation	Source of isolation	Year of isolation	No. of strains isolated
Bm 1M	*Bombyx mori*, gattine, dead silkworms, Czechoslovakia	1956	2
Bm 3M	*B. mori*, gattine, sick silkworms, Czechoslovakia	1956	1
Bm J1	*B. mori*, dead silkworms, Yugoslavia	1956	1
Bm B1(*)	*B. mori*, dead silkworms, Czechoslovakia	1956	1
Sc 3	*B. mori*, dead silkworms, Czechoslovakia	1957	4
Sc 2	*B. mori*, healthy silkworms, Czechoslovakia	1957	5

Streptococcus faecium NCTC 6783 (†)

Streptococcus faecalis S 197, isolated from a fly *Protophormia terraenovae*, Czechoslovakia, 1957.

* This strain was isolated by Dr Vankova.

† This strain was received as *S. faecalis*, but it was found that its biochemical features are the same as those given by Shattock (1955) and by Lake *et al.* (1957) for *S. faecium*.

and Lake *et al.* (1957) in which more modern methods for distinguishing enterococci are given.

The examination of pathogenicity of our strains was made on healthy silkworms, kept at laboratory temperature and fed with fresh washed mulberry leaves. For each experiment 25–50 silkworms were used. The infectivity of the streptococcal strains Bm 1M, Bm 3M, Bm J1, of *Streptococcus faecium* and of *S. faecalis* was tested by feeding the organisms to silkworms. Strain Bm 1M was also examined by injection into the body cavity of silkworms. Strains were preserved at 4° on agar slopes under medicinal paraffin oil.

The methods of infection were those used in earlier work (Weiser & Lysenko, 1956) with the difference that, instead of a glass capillary, a tuberculin syringe with fine intradermal needle was used to inject the body cavity of silkworms with 0·02 ml. of a suspension of streptococci (one loopful of 24 hr. culture from Tryptose agar suspended in 10 ml. 0·6 %, w/v, NaCl solution). Feeding experiments were made in the following way: silkworms of the third and fourth stage were fed with leaves infected with a heavy suspension of a streptococcal culture, the infected food being served only once to animals starved for one day.

In dead silkworms the presence of streptococci was proved by microscopical examination and by culture on blood agar. A short examination of physiological features of colonies resembling streptococci was made by the following tests: growth in the presence of 6·5 (w/v) NaCl; growth at pH 9·6; growth in the presence of potassium tellurite 1/2500; reduction of litmus milk; growth at 45°; fermentation of arabinose (for details of tests see literature cited). The localization of streptococci in the bodies of silkworms was examined on histological sections stained by Gram's method and counterstained with eosin Y 0·1 % (w/v) aqueous solution.

RESULTS

All the strains examined were typical enterococci by their biochemical and morphological features, the common features of our bombyx strains being as follows: good growth at 45°, and at pH 9·6; resisted heating at 60° for 30 min. These strains fermented glucose, mannitol, sucrose; gelatin was not liquefied. The strains were able to grow in the presence of 40 % (w/v) bile. They were not pathogenic for white mice on intraperitoneal injection. Other features, in which the bombyx strains differed from one another are given in Table 2, which also gives the features of bombyx strains reported by Seelemann (1942).

There were some features of the bombyx strains which were common with those of *Streptococcus faecalis* (inability to ferment arabinose) and on the other hand with those of *S. faecium* (glycerol not fermented anaerobically; citrates not used). The biochemical characters in which the bombyx strains differed from one another were:

(i) The reaction on blood agar was variable, some strains produced weak haemolysis, others showed all the variations between slight greening to classical α-haemolysis and others were frankly non-haemolytic.

(ii) Growth in the presence of 6·5% (w/v) NaCl was weak or nil; this was found also by Seelemann (1942).

(iii) Reduction of litmus milk: variable and not persistent; the maximum degree of reduction, if it appeared, might be observed after the incubation for 16 hr. but as a rule colour returned after two days.

(iv) Sorbitol was fermented slowly by most strains.

(v) Reduction of triphenyltetrazolium chloride: weak (pink colonies after 3 days) or negative.

(vi) Ability to grow in potassium tellurite 1/2500: weak (a small black sediment at bottom of test-tube) or absent.

On the basis of these observations it is clear that the bombyx strains examined were an ill-defined group of forms having affinities with *Streptococcus faecalis* and *S. faecium*. Differences were not found as between the bombyx strains isolated from sick or healthy silkworms.

Table 2. *Biochemical characters of bombyx strains examined*

All the bombyx strains studied here had the following characters: good growth at 45°, and at pH 9·6; resistant to 60° for 30 min.; glucose, mannitol, sucrose fermented; gelatin not liquefied; growth in presence of 40 % (w/v) bile.

	Bombyx strains						
	Bm 1M	Bm 3M	Bm J1	Bm B1	Sc 3	Sc 2	Seel.*
Blood agar, greening to α-haemolysis	(+)	(+)	−	−	(+)	(+)	+
Growth with NaCl 6·5 %	(+)5	+	(+)	+	(+)	−	−
Reduction of litmus milk	(+)	(+)	+	(+)	+	+	(+)
Acid from glycerol anaerobically	−	−	−	−	−	−	n.t.
Sorbitol	+5	+5	+5	+2	+5	+2	n.t.
Arabinose	−	−	−	−	−	−	n.t.
Utilization of citrates	−	−	−	−	−	−	n.t.
Potassium tellurite 1/2500	(+)	−	−	(+)	(+)3	(+)	n.t.
Tetrazolium reduction	(+)3	(+)3	(+)3	(+)3	+	(+)	n.t.

+ = Positive reaction; (+) = weakly positive; − = negative reaction; (the number beside the sign indicates the day on which reaction was observed); n.t. = not tested. Seel* = features of Seelemann's bombyx strains.

The results of infection experiments are given in Figs. 1 and 2. Figure 1 shows the mortality caused by feeding strains Bm 1M, Bm 3M and Bm J1, *Streptococcus faecalis* or *S. faecium*. Figure 2 gives curves indicating the mortality of silkworms after feeding and parenteral inoculation of strain Bm 1M. For comparison are given the results obtained by similar experiments with this strain made soon after its isolation in 1956, and those made after its preservation in the laboratory for a year. In Pl. 1, fig. 1, the typical diplococcal arrangement of streptococci in the gut of an infected silkworm is shown; Plate 1, fig. 2, shows a section of haemolymph of a dead silkworm containing streptococci and other bacteria, and the localization of streptococci in the gut (*G*) of a silkworm is shown in Pl. 1, fig. 3.

Thus, as may be seen, the streptococci were pathogenic for silkworms and the pathogenicity of bombyx strains was to some extent greater than that of the strains of *Streptococcus faecalis* and *S. faecium* used (Fig. 1). Infection by injection was more severe than the infection by feeding. After an incubation period of about 3 days the disease became apparent in injected silkworms and they began to die; within 7 days the mortality reached 100 %. With infection by feeding the incubation period was the same as by injection, but the disease then had a chronic character, and the mortality never reached 100 %; some silkworms survived the infection.

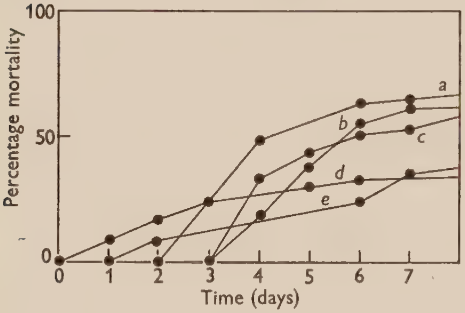

Fig. 1

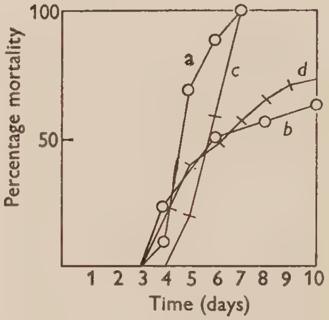

Fig. 2

Fig. 1. Death of silkworms after feeding strains: *a* = strain Bm J1; *b* = strain Bm 3M; *c* = strain Bm 1M; *d* = *Streptococcus faecium*; *e* = *S. faecalis*. Control deaths = 0.

Fig. 2. Death of silkworms after the infection by the strains Bm 1M. (*a*) by injection in the year 1957; (*b*) by feeding in the year 1956; (*c*) by injection in the year 1956; (*d*) by feeding in the year 1956. Mortality in controls in all experiments 5–10 % (not shown).

It is remarkable that the incubation period was similar by both routes of infection; according to my experience this is unusual with other microorganisms. I came to the conclusion that the streptococci did not cause a septicaemia but that the sickness was of gut origin. This I proved by histological examination; the streptococci were observed only in the gut and never in the body cavity or in the tissues of silkworms. Streptococci were found also in the haemolymph *post mortem* but they were there accompanied by other bacteria, especially pseudomonads, enterobacteria and staphylococci. My results are similar to those of Paillot (1930*a*, *b*,). It is also to be noted that for infection by feeding, large doses of infectious material must be used. It is also interesting that the virulence of the strain Bm 1M was not changed by its preservation under oil for a year.

DISCUSSION

From the experiments reported it can be stated that the bacteria isolated from the epizootics and designated *Streptococcus bombycis* were a heterogeneous group of streptococci; it was impossible to allocate them to the species *S. faecalis* or to *S. faecium*, with which they have affinities. On the other hand these strains could not be made into an independent species (*bombycis*) of the enterococcus group, because they showed great heterogeneity of biochemical features. According to Sneath (1957) such intermediate strains should be regarded as inter-forms and a separate term 'gephyron' was proposed for this purpose.

Michailov (1950) examined 298 strains of *Streptococcus bombycis*, which he considered to belong to the group of lactic streptococci, and concluded that these strains differed from one another in the action of litmus milk, on blood agar, in fermentation of mannitol and in temperature for optimal growth. Masera's (1954) strain of *S. bombycis* liquefied gelatin on prolonged incubation. These reports demonstrate the great variability of biochemical characters of strains named *Streptococcus bombycis* and may partly explain the differences which occur between various descriptions of *S. bombycis* and the many names and synonyms with which these organisms are connected. But an important reason for the mistakes no doubt was the static conception of former bacteriological taxonomy. The research worker in insect microbiology must in future use tests which enable the differentiation of bacteria inside the given group to be made; it is impossible to be satisfied with the generic designation without the corresponding species determination.

The species *Diplococcus bombycis* Paillot 1922 is, in my opinion, of doubtful validity, because its description was based on morphology only. The typical diplococcal arrangement of enterococci is quite common and I have also observed it in my strains (Pl. 1, figs. 1, 2). As mentioned before and by other workers (Paillot, 1930*a*, *b*, 1941; Steinhaus, 1949; Michailov, 1950; Masera, 1954) streptococci cause a disease of insect gut of chronic character, but under natural conditions some predisposing factors are necessary. It is known that the streptococci are only secondary invaders in virus diseases of insects under natural conditions (Steinhaus, 1949) and my experiments showed that large doses of streptococci, which silkworms would not meet under natural conditions, must be used to produce the disease. The mechanism of infection cannot be fully explained on the basis of my experiments. I suppose the great increase of streptococci in the gut to be connected with some factors such as virus infection or other influence able to stimulate the increase of streptococci in the gut which in silkworms causes a form of dyspepsia. The diverse biochemical activities and the resistance of the streptococci are the reasons for their persisting also in the gut of healthy silkworms. The pH range for growth streptococci is wide (pH 4·5–9·6) but the pH values which are necessary for the physiological functioning of insect gut are much more limited. By the multiplication of streptococci the pH value of the gut is lowered because in the gut contents are many substrates which can be fermented by streptococci. Streptococci as well as staphylococci are found in the gut rather more frequently than other bacteria, a fact which may be explained by the pH values of the gut. Michailov (1950) found pH 9·6–10, but Heimpel (1955, 1956) observed that the values depended on the period of starvation of silkworms and the values he obtained were lower than those of Michailov. My conception is confirmed by the fact that streptococci may be found not only in sick silk worms but also in healthy ones.

Because the pathogenicity of our bombyx strains does not differ essentially from the pathogenicity of the strains of *Streptococcus faecalis* and *S. faecium* examined, we may state that these bombyx strains are not essentially distinct from the streptococci which are widely distributed in nature. Under the name *S. bombycis* various atypical strains of enterococci may be listed; these are closely related to the species *S. faecalis* and *S. faecium* and, according to Masera's description, also to *S. faecalis* var. *zymogenes* or *S. faecalis* var. *liquefaciens*. For this reason I consider the organism named *S. bombycis*, synonyms *S. bombycis* (Pasteur) Flugge, *S. bombycis* Zopf, *S. bombycis* Sartirana & Paccanaro, *S. pastorianus* Krassilstschik, *Micrococcus bombycis* Cohn and *Diplococcus bombycis* Paillot, as insufficiently described and too variable to be regarded as a definite species. Therefore, the name *Streptococcus bombycis* should be rejected and should be included under *nomina rejicienda*.

I am indebted to Dr S. T. Cowan, to Academician Ivan Malek and to Dr Jaroslav Weiser for very helpful criticism and comments.

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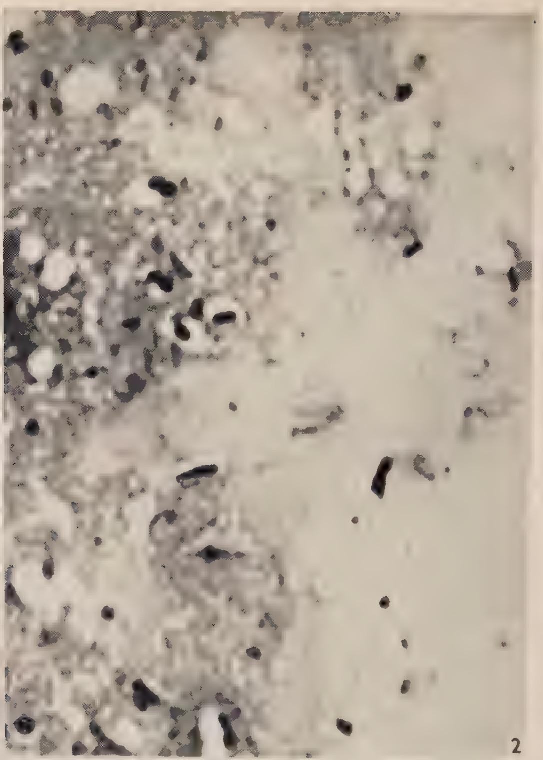

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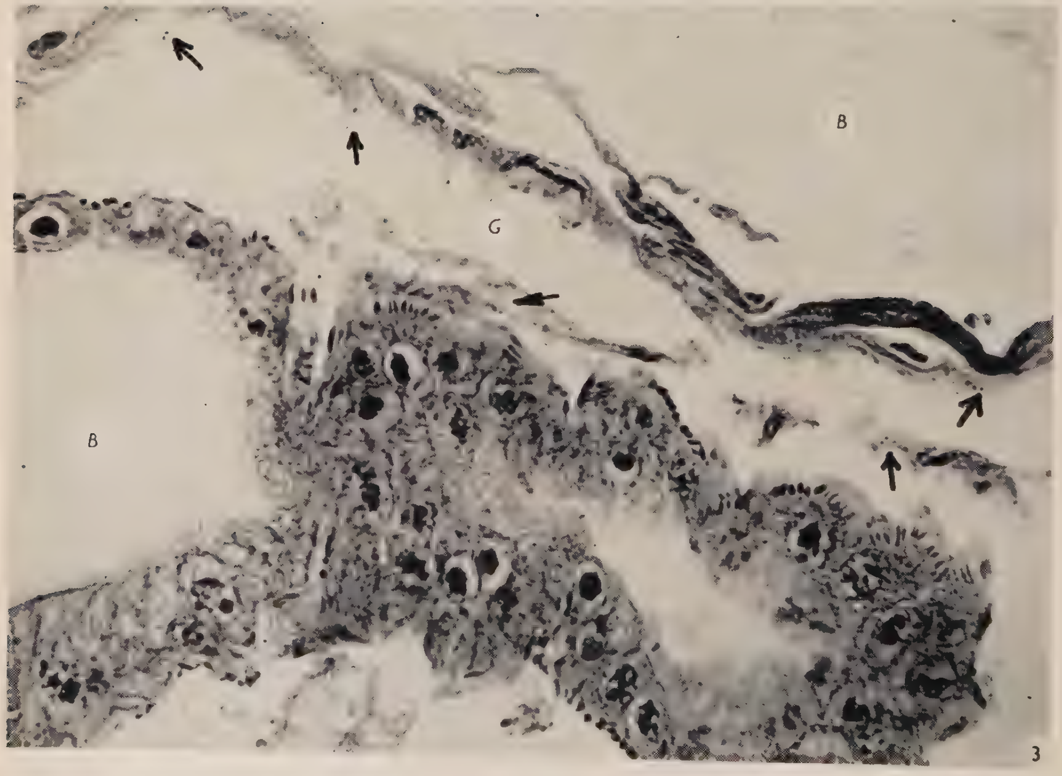

3

O. Lysenko—*Streptococcus bombycis.* Plate 1

(*Facing p.* 781)

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EXPLANATION OF PLATE

Fig. 1. *Streptococcus bombycis* strain Bm 1M in the gut of infected silkworm. × 4000.

Fig. 2. '*Streptococcus bombycis*' and other bacteria in the haemolymph of a dead silkworm in the stage of a partial decomposition caused by these bacteria. × 2500.

Fig. 3. Cross-section of silkworm gut; the localization of '*Streptococcus bombycis*' is shown by arrow heads. *G* = gut; *B* = body cavity. × 410.

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The Transfer of Motility and Tyrosine Requirement to *Escherichia coli* Strain B by Recombination with *E. coli* Strain K12

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SUMMARY: Motile recombinants may be obtained by crossing *Escherichia coli* strain K12, F+, with the non-flagellated strain *E. coli* strain B, F−. The flagella of the recombinants have the same antigenic pattern as those of the F+ parent and the locus controlling motility is linked to loci controlling tyrosine requirement and fermentation of arabinose and galactose (gal_5).

Motility may be transduced to non-motile strains of Salmonella by phage grown on motile Salmonella strains (Stocker, Zinder & Lederberg, 1953). The majority of the motile clones thus obtained produce flagella having the antigenic composition of the motile parent stock from which the non-motile recipient strain was derived by mutation. Transduction therefore normally reveals their inherent antigenic pattern. By using suitable selective techniques, in some cases, a minority of motile recombinants having flagella of the antigenic pattern of the bacteria on which the phages were propagated (donor strain) can be demonstrated.

These observations suggested that motility might similarly be transferred by recombination between mutants of *Escherichia coli* strain K12 and mapped as another marker on the bacterial chromosome. However, all mutants of strain K12 were found to become motile on inoculating Craigie tubes. *E. coli* strain B, on the other hand, never mutated to motility but acquired motility when incubated with motile strain K12 F+ mutants (Furness & Rowley, 1955). All the motile progeny had flagella of the same antigenic pattern as the F+ parent but linkage with other characters could not be shown. Linkage has been further investigated by using auxotrophic strains of *E. coli* strain B. This paper reports the results of experiments in which it has been found that motility is linked to other markers on the bacterial chromosome, namely tyrosine requirement and ability to ferment arabinose and galactose.

METHODS

Strains of Escherichia coli *strain B.* Both strains were F−, non-motile, resistant to 500 μg. streptomycin/ml., fermented lactose, arabinose, galactose, xylose and mannitol but not maltose (lac^+, $arab^+$, gal^+, xyl^+ mtl^+, mal^-) and were sensitive to all of the T series of phages. *E. coli* B/S^RF− grows on minimal medium (Davis & Mingioli, 1950). *E. coli* B mutant 615/S^RF− was kindly made available to us by Dr de Haan and was auxotrophic for tyrosine and methionine (de Haan, 1955).

Strains of Escherichia coli *K* 12. Strain W677 was auxotrophic for threonine, leucine and vitamin B_1 ($T^-L^-B_1^-$), did not ferment lactose, arabinose, galactose, maltose, xylose and mannitol (*lac*$^-$, *arab*$^-$, *gal*$_5^-$, *mal*$^-$, *xyl*$^-$, *mtl*$^-$) and was resistant to phages T_1 and T_5 ($V^R_{1,5}$; Clowes & Rowley, 1954; Lennox, 1955). It was streptomycin sensitive and F+, mating with *E. coli* B strains. To ensure that all organisms were fully motile it had been repeatedly passaged through Craigie tubes.

Media. Minimal medium was that of Davis & Mingioli (1950) and the formula of Lederberg (1950) was used for eosin+methylene-blue sugar-containing media. The minimal medium was supplemented with 20 μg./ml. of tyrosine or methionine when required. Craigie tubes were prepared as described by Tulloch (1939) using nutrient broth solidified with 0·5% Difco agar. Semi-solid gelatin agar was made by the method of Stocker *et al.* (1953). All streptomycin media contained 500 μg. streptomycin/ml.

Recombination techniques. Using overnight cultures 3 ml. of coli B parent and 6 ml. F+ parent were mixed, incubated ½ hr. at 37°, diluted with 45 ml. broth and incubation continued for a further 3½ hr. 8 ml. of the mixture and 5 ml. of each parent, as controls, were centrifuged. Further stages of the technique depended on the recombinants to be selected, as follows.

(*a*) *Selection of motile recombinants.* The deposits after centrifugation were spread by loop in arcs (Stocker *et al.* 1953) on plates of semi-solid gelatin agar containing streptomycin to eliminate the F+ parent. The plates were incubated at 37° for 24 hr. and left at room temperature for a further 72 hr.

(*b*) *Selection of mono-auxotrophic recombinants.* The organisms were washed three times in minimal salts, resuspended in approximately one-tenth the original volume and 0·1 ml. amounts were spread on minimal medium plates supplemented as necessary. All plates were incubated at 37° for 42 hr.

Purification of recombinants. Motile or mono-auxotrophic recombinants were purified by picking a discrete colony after plating out on nutrient agar. After checking that the purified strain still showed the recombinant characters, it was tested for the unselected markers by standard methods for testing sugar fermentations, streptomycin resistance, phage resistance and nutritional requirements; motility was tested by microscopic examination and ability to grow through a Craigie tube within 24 hr.

'*H*' *agglutination technique.* Agglutinating serum obtained from a rabbit immunized with a saline suspension of motile *Escherichia coli* strain K12 was inactivated by heat. Standard methods were used for the preparation of 'H' and 'O' agglutinable suspensions and for the Dreyer agglutination test (Mackie & McCartney, 1948).

RESULTS

Recombination using motility as selective marker of the F+ parent

Ninety-eight motile streptomycin-resistant recombinants from the mating W677 F^+ × Coli B/S^RF− were picked from discrete flares in the semi-solid streptomycin gelatin agar and tested for the unselected markers of the F+ parent, i.e., T^-, L^-, B_1^-, $V^R_{1,5}$, *lac*,$^-$ *arab*$^-$, *gal*$_5^-$, *xyl*$^-$, and *mtl*$^-$. These recombinants had all the unselected markers of the F− parent. Control plates of semi-solid gelatin agar containing streptomycin when inoculated with W677 F+ alone showed no growth and when inoculated with coli B/S^RF− alone never yielded flares, indicating that the F+ parent had not mutated to streptomycin resistance, nor the F− parent to motility.

'H' agglutinable suspensions were prepared from these recombinants and from the strain K12 culture used for the preparation of the agglutinating serum. All suspensions including the K12 suspension agglutinated to the same titre with the serum prepared against the motile K12 parent; this showed that the flagella of the recombinants and of the F+ parent had the same antigenic composition. Using this technique, neither 'O' agglutinable suspensions of K12 nor saline suspensions of coli B/S^R agglutinated with this serum.

On mating W677 F+ and 615S^RF− by the same method it was found that the unselected marker tyr^+ had been transferred from the F+ parent to 10·7 % of the 84 motile, streptomycin-resistant recombinants tested. One of these motile tyr^+ recombinants was also $arab^-$, gal_5^- (see Table 1). No other marker from the F+ parent was found in any of the recombinants. It is concluded that *motility* is linked to *tyr* and, less closely, to *arab* and gal_5.

Table 1. *Transfer of unselected markers*

From F+ parent to recombinants on mating *E. coli* W677 F+ motile ($T^-L^-B_1^-V^R_{1,5}$, lac^-, $arab^-$, gal^-, xyl^-, mtl^-) with *E. coli* B615S^RF− non motile (Tyr^-, M^-, lac^+, $arab^+$, gal^+, xyl^+, mtl^+) on streptomycin semi-solid gelatin agar using motility as selective marker of the F+ parent

Unselected F+ markers transferred				
tyr^+	$arab^-$	gal^-	No. of recombinants	%
−	−	−	74	88·1
+	−	−	9	10·7
+	+	+	1	1·2
Total motile recombinants			84	

+ Indicates transfer of unselected markers from F+ parent. The unselected markers $T^-L^-B_1^-V^R_{1,5}$ lac^-, xyl^-, mtl^- were not transferred from the F+ parent to any of the recombinants total.

The rate of recombination was estimated at 1 motile recombinant in 10^8 *Escherichia coli* strain B cells, but an accurate determination could not be made by the technique of streaking centrifuged deposits on plates.

Recombination using tyr^+ as selective marker of the F+ parent

Samples of a mixture of W677 F+ and 615S^RF− were spread on minimal medium supplemented with methionine, and also on minimal medium supplemented with methionine and streptomycin (to examine the effect of contraselection for streptomycin-sensitivity). It was found that the inclusion of streptomycin reduced the number of recombinants from 1 in 5×10^5 615S^RF− cells to 1 in 5×10^6. Control plates seeded with either W677F+ or 615S^RF− alone gave no colonies.

On testing the unselected markers of the recombinants recovered from the methionine minimal plates it was found that only 3·9 % of the recombinant clones were streptomycin-sensitive, which suggests that contra-selection was not the sole reason for the reduction in the yield of recombinants when streptomycin was included. Moreover, the presence of streptomycin not only reduced the yield of tyr^+ recombinants but, with the exception of *motility*, also significantly effected the relative frequency of the various classes of recombinants (see table 2).

Table 2. *Transfer of unselected markers*

From F+ parent to recombinants on mating *E. coli* W677S^S motile F^+ ($T^-L^-B_1^-$, $V^R_{1,5}$ lac^-, $arab^-$, gal_5^-, xyl^-, mtl^-) with *E. coli* B615S^RF− non-motile (Tyr^-, M^-, lac^+, $arab^+$, gal^+, xyl^+, mtl^+) on minimal medium supplemented with methionine, or methionine and streptomycin, tyr^+ being the selective marker of the F+ parent.

Unselected markers transferred from F+ parent									Supplements to medium			
									Methionine		Methionine and streptomycin	
$V^R_{1,5}$	lac^-	motility	$arab^-$	gal_5^-	S^S	xyl^-	mtl^-	M^+	No.	%	No.	%
−	−	−	−	−	−	−	−	−	80	62·5	124	72·5
−	−	−	+	−	−	−	−	−	9	7·0	6	3·5
−	−	−	+	+	−	−	−	−	10	7·8	8	4·6
−	−	−	−	+	−	−	−	−	1	0·8	3	1·8
−	−	+	−	−	−	−	−	−	9	7·0	25	14·6
−	−	+	+	−	−	−	−	−	5	3·9	3	1·8
−	−	+	+	+	−	−	−	−	7	5·4	2	1·2
+	+	+	+	+	+	+	+	+	1	0·8	.	.
−	−	−	+	+	+	+	+	+	1	0·8	.	.
+	−	+	+	+	−	−	−	−	1	0·8	.	.
+	+	−	−	−	−	−	−	−	1	0·8	.	.
−	−	−	−	−	+	+	−	−	1	0·8	.	.
−	−	−	−	−	+	+	+	−	1	0·8	.	.
−	−	−	+	+	+	+	−	+	1	0·8	.	.
Total recombinants tested									128		171	

+ indicates transfer of unselected marker from F+ parent.

When tyr^+ was used as the selective marker from the F+ parent the only frequent (> 2 %) class of recombinants obtained were: those with no unselected marker from the F+ parent (62·5 % or 72·5 %); those with the unselected markers *motility* (7 % or 14·6 %); *arab* (7 % or 3·5 %); *arab* and gal_5 (7·8 % or 4·6 %); *motility* and *arab* (3·9 % or 1·8 %); *motility, arab* and *gal* (5·4 % or 1·2 %); the percentages for each class being stated for recombinants selected in the absence and in the presence of streptomycin. These frequent classes can be explained as resulting from double, rather than multiple, cross-overs in the region of the *tyr* locus if the order of the loci concerned is *motility*–*tyr*–(*arab*, gal_5), with none of the other unselected markers tested lying between *motility* and (*arab*, gal_5).

DISCUSSION

Electron micrographs have shown that the cells of *Escherichia coli* strain B are without flagella and attempts to obtain motile mutants have failed. As a result, it is impossible to find the latent flagellar antigens, if any, of *E. coli* B for comparison with those of *E. coli* K12. The flagella of the motile progeny have the antigenic pattern of K12, the F+ parent. This is in contrast to the salmonellas, where, when motility is transduced to a non-flagellated strain, the latent genes determining the H antigens of the recipient strain are usually expressed.

From the present data the loci concerned appear to be in the order *motility–tyr–(arab, gal_5)*. De Haan (1955) using the streptomycin-sensitive parent of this 615S^RF− strain has reported that the linkage results in recombination experiments can be markedly altered by the cultural conditions of the F− strain and also by the addition of methionine to the medium used for selecting recombinants. It is possible that streptomycin has a similar effect. The action of added streptomycin, in altering the apparent closeness of linkage of *arab* and of *gal_5* to *motility* and *tyr*, cannot at present be explained.

I wish to thank Dr P. G. de Haan for the auxotrophic mutants of *Escherichia coli* strain B, Dr D. Rowley for his interest and encouragement and Miss Margaret Ham for her excellent technical assistance.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its twenty-fifth General Meeting in the University of Cambridge on Monday and Tuesday, 23 *and* 24 *September* 1957. *The following communications were made:*

COMMUNICATIONS

The Properties of a Gram-Negative Coccus from the Rumen of the Calf.

By P. N. Hobson, S. O. Mann and A. E. Oxford (*The Rowett Research Institute, Bucksburn, Aberdeenshire*)

The coccus was first isolated on tomato-extract medium from the rumens of young calves. Subsequent isolations were made on a lactate containing medium. The organism was present in a 5-day-old calf and was found in numbers of up to 10^{10}/ml. rumen contents in a number of other young calves on milk or concentrate diets. It was not isolated from one 4-month-old calf (and only at 10^2/ml. from another), and not from six heifers, or two sheep on hay or concentrate diets.

The coccus is $1\cdot5\mu$. diameter, generally alone, or in diplococcal forms with flattened adjacent sides, or in groups; chains are rare. It is an obligate anaerobe, and ferments glucose, sucrose, maltose, fructose, sorbitol, mannitol to acid and gas. It ferments lactate, but not fumarate, formate, succinate, malate or alanine. It is not amylolytic or iodophilic, H_2S is formed, but not indole, catalase or oxidase, and gelatin is not liquefied.

From lactate the organism forms mainly acetic, propionic, butyric and valeric acids, and from glucose acetic, butyric, valeric and caproic.

From the 211 isolations tested three distinct serological types, with a possible fourth, have been obtained. By the use of fluorescent antisera, and by tests on isolated organisms, the occurrence of these types in the rumen has been investigated. In some calves one type alone was found early in life, but this changed to a mixture of types, or was replaced by another during the course of a few weeks.

The organism is similar to one isolated from sheep by Elsden, Volcani, Gilchrist & Lewis (1956).

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Antigenic Distinctions between Moulds in Ouchterlony gel Plates.

By J. G. Feinberg and Hazel Grayson (*Bencard Allergy Research Unit, Beecham Research Laboratories, Ltd., Betchworth, Surrey*)

Specific diagnosis and treatment of mould allergies is based on the use of mould extracts. To reduce diagnostic complexity it is common practice to assume broad antigenic relationships between moulds, especially between species within the same genus. Such assumption has proved reasonably valid among the common grass pollens, but is so far hardly tested in the moulds. The current investigation is designed to study the antigenic relationships of moulds through their reactions with homospecific and heterospecific rabbit antisera in agar gel plates.

At this stage the study has covered common species of seven genera: *Penicillium* (*brevicompactum*), *Neurospora* (*sitophila*), *Botrytis* (*cinerea*), *Aspergillus* (*nidulans* and *amstelodami*), *Mucor* (*racemosus*), *Rhizopus* (*nigricans*), *Cladosporium* (*herbarum*). The two Aspergilli serve as a preliminary look at two species within the same genus.

In the main, with exceptions to be noted, antigenic relationships between the moulds so far studied are either absent or unremarkable. Even between the two Aspergilli there is virtually no cross-reaction, nor is there any apparent antigenic relationship between the cytologically closely related genera, *Penicillium* and *Aspergillus*.

On the other hand, one prominent antigen has been found common to *Mucor racemosus* and *Rhizopus nigricans*. It is noteworthy that these are the only two Phycomycetes in the present series. The Phycomycetes characteristically have a hydrophylic substance in their sporangia and it is tentatively suggested that this substance may be the common antigen observed in the gel plates.

An unexpected antigen-antibody band was observed between the *Mucor racemosus* mould suspension and the *Cladosporium herbarum* antiserum. No explanation for this is apparent on a taxonomic basis.

The authors are indebted to Dr M. Richards, of these Laboratories, for advice and guidance on mycological matters.

The Influence of Growth Rate and Aeration on the Respiratory System of a Fluorescent Pseudomonad, strain KB1, Grown in Continuous Culture. By R. F. Rosenberger and Margot Kogut, A.R.C. (*Unit for Microbiology, The University, Sheffield* 10)

The technique of continuous culture provides the means of studying single factors governing growth and synthetic activities of micro-organisms. Thus, studies concerning the effects of oxygen supply on bacteria should satisfy the following requirements: First, the amount of oxygen per cell should be constant throughout the experiment. Secondly, the growth rate should not be affected by the variation in oxygen supply. Thirdly, it is desirable to have the cells in the exponential phase throughout. All these conditions can be fulfilled in continuous culture.

A chemostat-type of continuous culture apparatus was used, and the organism was a strictly aerobic fluorescent Pseudomonad, strain KB1. It was grown at 25° on a simple mineral medium with succinate as sole source of carbon, at varying growth rates controlled by the medium flow. At each growth rate, the carbon source and air supply were alternately made the factors governing cell density. From each steady state samples were taken and cell density determined directly and turbidimetrically. Washed suspensions prepared from such samples were examined for oxidizing ability by measuring the Q_{O_2} succinate and for cytochrome content by a method based on that described by Shibata, Benson & Calvin (1954).

The results showed that the Q_{O_2} succinate was not affected by variation in growth rate, whilst it was perhaps slightly decreased by growth under air-limiting conditions. Cytochrome content, on the other hand, was affected by both these factors. Cells grown under air-limiting conditions had approximately double the cytochrome content of cells grown under succinate-limiting conditions; and for each of these conditions cytochrome increased with decrease in growth rate.

These results are compared with those of Chaix & Petit (1956) and Lenhoff & Kaplan (1956) who have studied the effects of growth conditions on production of cytochromes in batch cultures of *Bacillus subtilis* and *Pseudomonas fluorescens* respectively.

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Amino Acid–Amino Sugar–Lipid Complexes Accumulating in the Growth Medium of an *Escherichia coli* Mutant.

By Pauline Meadow (*Department of Biochemistry, University College, London, W.C.* 1)

The lysine-deficient mutant *Escherichia coli* 26–26 accumulates diaminopimelic acid in its growth medium when grown in low concentrations of lysine (10 mg./l.). Boiling the culture filtrate at pH 3·8 precipitated a substance which was hydrolysed with 6 N-hydrochloric acid for 18 hr. and investigated chromatographically. It contained all the normal protein amino acids as well as diaminopimelic acid, glucosamine, ethanolamine, glucose and galactose. The conditions necessary for its production were those required for diaminopimelic acid accumulation and included good aeration, low lysine concentration and a temperature of 28°–37°. The amount produced increased from 72 to 120 mg./l. in 24–40 hr. incubation during which time the turbidity of the suspension increased.

Part of this lipomucoprotein was extracted by freezing at $-12°$ in the presence of ether, the remainder being precipitated by boiling or half saturating with ammonium sulphate at pH 3·8. The frozen out and precipitable fractions were qualitatively similar in composition, but the former contained 40% ether-extractable lipid, while the latter had the 20% lipid content characteristic of coli cell walls.

The two lipoproteins and a purified preparation of cell walls of the mutant were extracted with 90% phenol and the extracts and residues hydrolysed and investigated chromatographically. The phenol-soluble portions contained most amino acids, glucosamine and ethanolamine but no diaminopimelic acid, muramic acid or sugars. The phenol-insoluble residues contained chiefly aspartic and glutamic acids, lysine, and alanine together with glucose and galactose but no glucosamine. This fraction of the cell walls contained muramic acid. Apart from the absence of muramic acid the lipoproteins resembled coli cell walls and may represent abortive attempts to build cell walls.

Mechanism of Action for Nitrite Reductase from *Neurospora crassa*.

By D. J. D. Nicholas. (*Agricultural Research Council Unit of Plant Nutrition (Micronutrients), Long Ashton Research Station, University of Bristol*)

A nitrite reductase from *Neurospora crassa*, wild type 5297*a* (macroconidial) is a flavoprotein dependent on Cu and Fe for its activity and requiring DPNH as the electron donor (Medina & Nicholas, 1957*a*, *b*). The enzyme which is particulate was stable in 10% (w/v) sucrose and was rendered soluble with deoxycholate. The K_m of the purified enzyme-NO_2^- complex was $1{\cdot}5 \times 10^{-3}$ moles NO_2^-/l. Hyponitrite, the product of the enzyme, inhibited its activity whereas the addition of hydroxylamine was without effect.

Cytochromes *b* and c_1 were identified in the purified enzyme. DPNH reduced the bound-FAD peak, at 430 mμ., in the enzyme, and the haem peaks appeared at 553, 562 mμ. The reduction of haem-Fe with DPNH was more pronounced when flavin as boiled pig-heart or as FAD, was added to the enzyme. Thus flavin precedes haem-Fe in the reduction sequence. The addition of NO_2^- markedly reduced the α peaks of cytochromes *b* and c_1, thus demonstrating that reduced haem is oxidized by NO_2^-. Cu_2Cl_2, prepared from $CuCl_2$ by reduction with copper turnings just prior to use, served as an electron donor for the enzymic reduction of nitrite; the addition of flavin had no effect on this part of the system. Cu^{1+} failed to reduce haem-Fe in the enzyme, thus the latter precedes Cu^{1+} in electron transport. The sequence of electron transfer in nitrite reductase is probably:

$$\text{DPNH} \rightarrow \text{FAD} \rightarrow \text{cytochrome } b \rightarrow \text{cytochrome } c_1 \rightarrow Cu^{1+\rightarrow 2+} \rightarrow NO_2^- \rightarrow N_2O_2^{--}.$$

There is a phosphorylation during nitrite reductase action because uncoupling reagents inhibited the enzyme and inorganic P was incorporated into ATP.

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Inhibitors of Protein and Nucleic Acid Synthesis in *Aspergillus nidulans*.

By C. J. Shepherd (*M.R.C. Unit for Chemical Microbiology, University of Cambridge*)

Investigations on the effect of *p*-*N*-di(chloroethyl)phenyl-butyric acid (CB 1348) and L-*p*-*N*-di(chloroethyl)phenyl-alanine (CB 3025) on the synthesis of nucleic acid and protein by germinating conidia of *Aspergillus nidulans* have shown that, at a concentration of 2×10^{-6}M, both these compounds completely inhibit protein synthesis. With CB 1348, the synthesis of nucleic acid, although reduced, can continue for a further 6 hr. Upon separation of the nucleic acid by the method of Ogur & Rosen (1950) it was found that the residual synthesis was due to the synthesis of ribonucleic acid (RNA) only and that deoxyribonucleic acid (DNA) synthesis was completely inhibited. In the case of CB 3025, the synthesis of RNA was again unaffected, while DNA synthesis was reduced to approximately half that occurring in the absence of the inhibitor. Neither of the above compounds had any significant effect on respiration, or the synthesis of free amino acids and nucleotides, but both compounds produced a marked inhibition of the synthesis of cell-wall material.

Similar investigations showed that the antibiotic, actidione, at a concentration of $3{\cdot}5 \times 10^{-5}$ M, had similar effects to CB 1348, the experimental results being comparable with those obtained by Kerridge (1957) in a study of the effect of actidione on protein and nucleic acid synthesis in *Saccharomyces mandshuricus*.

The antibiotic, gliotoxin, at a concentration of 2×10^{-5} M, produced complete inhibition of protein and RNA synthesis, while DNA synthesis was virtually unaffected for a period of 3 hr. following the addition of the antibiotic.

With both actidione and gliotoxin, synthesis of cell-wall material was markedly inhibited.

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Release and Analysis of the Intracellular Amino Acids of *Staphylococcus aureus*. By R. Hancock (*Department of Biochemistry, University of Cambridge*)

Washed cells from exponentially growing cultures of *Staphylococcus aureus* (in the medium of Gale (1947)) contain *c.* 400 μmole (glutamic acid equivalents)/g. of ninhydrin-reacting material, estimated by the method of Cocking & Yemm (1954), over 90 % of which consists of amino acids, Using a membrane-filter technique it has been found that complete release of these amino acids occurs in trichloroacetic acid (TCA) (5 %, w/v), perchloric acid (0·2 N), *n*-butanol (10 %, v/v), ethanol (25 %, v/v), 0·1 N-HCl and 0·1 N-NaOH in 10 min. at 5°; in cetyltrimethylammonium bromide (100 μg./ml.) in 30 min. at 37° and in *NN*-dimethylformamide in 20 min. at 5°. All these reagents extract identical amounts of amino acids; extraction by *NN*-dimethylformamide (Dorough & Seaton, 1954) gives relatively salt-free fractions suitable for paper chromatography.

All amino acids which are present in the cell protein, with exception of tryptophan, are found on paper chromatograms of this amino acid 'pool'; no glucosamine is detectable. Quantitative analysis on columns of Dowex 50 (Moore & Stein, 1954) shows that over 97 % of the ninhydrin-reacting material is present in 19 amino acid components and ammonia; the quantity of TCA-soluble peptides, if present, must be less than *c.* 8 μmole/g. Glutamic and aspartic acids, proline, methionine, isoleucine and alanine are the major components.

Concentration gradients of the same order of magnitude as those attained for glutamic and aspartic acids (Gale, 1953) are maintained for methionine and isoleucine; the gradient for proline is more than twice that for glutamic acid.

One unidentified component, observed in fractions extracted by all methods, appears to be a small dialysable basic peptide; on hydrolysis (6N-HCl, 105°, 16 hr.) glutamic acid, glycine, alanine and lysine, with traces of aspartic acid, are released.

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The Change in Activity of Amino Acid Oxidases in Aged Suspensions of a *Proteus* sp. By Patricia H. Clarke (*Biochemistry Department, University College, London*)

Freshly harvested suspensions of *Proteus* species oxidatively deaminate almost all naturally occurring amino acids. Most of the monocarboxylic monoamino acids are oxidized by enzymes stable at 0° for many weeks in whole cells and cell-free preparations. The dicarboxylic and diamino acids (except arginine) are oxidized by labile enzymes. A washed suspension of a Providence culture NCTC 9400 with an initial Q_{O_2} glutamic acid of 150 lost 90 % of its activity after ageing for 4 weeks at 0°. The initial enzyme activity was reduced to one-third after 2 weeks at 0°, 24 hr. at 37° or freezing and thawing once, and almost completely lost after 48 hr. at 37° or freezing and thawing twice. Incubation of aged suspensions with glutamic or aspartic acids or proline resulted in a rapidly increasing rate of oxidation, and the recovery was completely inhibited by chloramphenicol. The increased rate of glutamic acid oxidation was accompanied by an increase in the number of viable cells in the suspension. Freeze-dried cells and cell-free preparations had no basal enzyme and no recovery. The rate of recovery is dependent on the basal enzyme level and not on the number of viable cells in the treated suspension.

The Electron Microscopy of *Streptomyces coelicolor*. By D. A. Hopwood and Audrey M. Glauert (*Botany School, University of Cambridge and Strangeways Research Laboratory, Cambridge*)

Electron micrographs of thin sections of *Streptomyces coelicolor* colonies embedded in methacrylate have shown that the mycelium has a structure comparable with that of other bacteria. It has, however, a greater cytoplasmic complexity. In the hyphae the nuclear material appears as an axial dense structure consisting of a number of threads and tubes. No nuclear membrane is observed. The hyphae also contain electron-dense polymetaphosphate granules and small laminated bodies which are possibly sites of formation of these granules. There are discrete regions of the cytoplasm which have an organized structure reminiscent of the endoplasmic reticulum of the cells of higher organisms.

Stages in the division of the nuclear material of the aerial mycelium during spore-formation have been studied. Different stages can be placed objectively in order by reference to stages in delimitation of the spores. Each mature spore has a central nuclear body consisting of an outer region of strands of approximately 200 Å diameter surrounding a less electron-dense core.

Observations on the Fine Structure of the Rous Virus. By M. A. Epstein (*Bland-Sutton Institute of Pathology, London*)

Observations on some Molar Growth Yields of Bacteria and Yeasts. By T. Bauchop (*A.R.C. Unit of Microbiology, Department of Microbiology, University of Sheffield*)

The molar growth yield (MGY) of an organism growing on a given substrate is defined as MGY = μg. dry wt. of cells/μmole of substrate utilized. The MGY's of *Streptococcus faecalis* and of *Saccharomyces cerevisiae* grown anaerobically on glucose as energy source were 22 and 21 μg./μmole respectively. *Streptococcus faecalis* grown anaerobically on D-ribose gave an MGY of 20 μg./μmole. Supplementation of the glucose-containing medium with L-arginine gave increased growth of *S. faecalis* and the growth was proportional to the arginine added. The MGY from arginine was 10 μg./μmole.

It is known that both *S. faecalis* and *Saccharomyces cerevisiae* metabolize glucose anaerobically by the Embden–Meyerhof pathway, a process which yields 2 mole of adenosine triphosphate (ATP)/mole glucose fermented. The recent work of Horecker and his colleagues suggests that when pentoses are fermented by homofermentative lactic acid bacteria, 2 mole of ATP are formed per mole of pentose fermented. Knivett has shown that during the conversion of arginine to ornithine by the 'arginine dihydrolase' system, 1 mole of ATP is formed per mole of arginine. If these yields of ATP from these substrates are also obtained *in vivo*, then it is possible to calculate the μg. dry wt. cells/μmole of ATP formed, and in this way relate growth to the available energy. *Streptococcus faecalis* gave yields of 11, 10 and 10 μg. dry wt./mole ATP when grown on glucose, D-ribose and L-arginine respectively. *Saccharomyces cerevisiae* gave a yield of 10·5 μg. dry wt./mole ATP when grown on glucose.

The MGY of *Propionibacterium pentosaceum* growing anaerobically on glucose was 37 μg. dry wt./mole glucose, a value almost double that from both *Streptococcus faecalis* and *Saccharomyces cerevisiae* growing anaerobically on the same substrate. When *Propionibacterium pentosaceum* was grown on lactate and glycerol the yields were 7·7 and 20 μg. dry wt./μmole respectively.

The Effect of Growth-rate on the Anatomy of *Escherichia coli*. By C. J. Perret (*Guinness-Lister Unit, Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W.* 1)

Escherichia coli K12 was grown at 36°, in both homocontinuous and regularly diluted batch cultures, at known constant doubling times between 22 and 345 min., with oxygen, glucose or unknown broth components as limiting nutrients in different experiments. Samples killed with 10% formalin were mounted on thin layers of 1·5% agar, examined under the phase-contrast microscope, and photographed. For every sample the number of transverse light bands shown by each of 600–1600 bacteria in a series of fields selected at random was recorded by direct observation. Bacterial dimensions were determined by measuring the photographic images projected on a translucent screen at a final magnification of 10^4.

Analysis of the data disclosed that the mean distance, d, between the centres of pairs of successive dark bands was constant at $1{\cdot}57 \pm 0{\cdot}13\mu$. ($P = 0{\cdot}05$) for all growth rates tested. The mean time interval, T, between inception of a dark band and cell division through that band, was found to be comparatively independent of growth rate; being related to the population doubling time, D, by the approximate expression

$$T = 34 + 0{\cdot}088D \text{ min.} \tag{1}$$

For a population of bacteria dividing asynchronously, with all individuals having the same constant doubling time and lengthening exponentially throughout their life spans, the following relationships should apply,

$$F = e^{\ln 2T/D}, \tag{2}$$

$$L = Fd, \tag{3}$$

where F and L are, respectively, mean number of light bands per cell, and mean cell length. Substituting the determined values of T, D and d in equations (2) and (3) gave strikingly accurate forecasts of F and L for all growth rates tested. If a light band is, or contains, a single 'chromatin body', the results suggest, amongst other things, an interesting virtual independence between the times required for cell doubling and for the division of chromatin bodies.

Toxic Action of Adenovirus. By H. G. Pereira and Barbara Kelly

(*National Institute for Medical Research, Mill Hill, London*)

Site of Zygote Formation in *Escherichia coli*. By Helen L. Bernstein

(*The Lister Institute of Preventive Medicine, London*)

In a mating pair, the cell which is the site of zygote formation is termed the recipient parent and the other, the donor. To see whether donor or recipient capacities were correlated with mating type, two kinds of experiments were done: (1) parents were killed with streptomycin, then mated; (2) parents were mated 60 min., then streptomycin was added to kill one parent and any zygotes contained therein. Broth cultures of *Escherichia coli* K-12 mutants ($M-F+$, $F-$; $T-L-B_1-F+$, $F-$) were treated 60 min. with streptomycin 1000μg./ml., then centrifuged, washed twice, viable counts were made, and cells were plated on minimal medium containing B_1.

(1) When treated log phase (2×10^7 cells/ml.) cultures were mated to untreated cultures, they were completely infertile regardless of mating type. But treated non-growing cultures (2×10^9 cells/ml.) of all mating types were

still fertile to some extent with untreated cultures. Thus, streptomycin prevents growing cells acting as either donors or recipients; but treated non-growing cells are still able to mate, and being non-viable can presumably only act as donor parents.

(2) When mated parents were treated in log phase, F− acted solely as recipients, F+ solely as donors, in F+ × F− crosses; in F+ × F+ crosses each parent acted as donors and as recipients. In stationary phase all mating types were both donors and recipients.

With a foreign F agent (F3) the same results were obtained. When K-12 mutants were mated to *Escherichia coli* strains of different mating types, all mating types acted in both capacities.

Since all mating types investigated could act to some extent as both donor and recipient parent, there is no absolute correlation between mating type and donor or recipient capacity. It was also noted that the recipient parent does not necessarily contribute the majority of unselected genetic markers.

Lysogenic Conversion by the A Phages of *Salmonella typhimurium*. By B. A. D. Stocker (*Lister Institute of Preventive Medicine, London*)

The heat-stable A phages obtainable from lysogenic *Salmonella typhimurium* strains (Boyd, 1954) have been examined for their 'converting' abilities. All the phages of group A1–A2, but not the A3 and A4 phages, effect O antigen conversion (Iseki & Kashiwagi, 1955): when they lysogenize *S. typhimurium* strains lacking somatic antigen **1** of the Kauffmann–White scheme, they cause this antigen to appear. Every plaque yields secondary growth agglutinable by factor **1** serum; therefore every phage particle can effect the change, which must be a necessary consequence of the lysogenization (in contrast to genetic transduction). Newly lysogenized clones are strongly agglutinable by factor **1** serum, but in established lysogenic cultures factor **1** shows 'form-variation', most colonies containing only a minority of agglutinable bacteria: it appears that lysogenicity confers the potentiality of producing antigen **1**, but that only a proportion of lysogenic cells manifest the antigen. Nearly all tested *S. typhimurium* strains having antigen **1** were lysogenic, yielding phage which induced this antigen. Agglutinability by factor **1** serum is detectable well before the end of the latent period of multiplication of either temperate or virulent phage; the change is therefore 'phage conversion' rather than 'lysogenic conversion'.

Lysogenization with A3 and A4 phages convert in respect of phage-absorbing capacity (Boyd, 1954): when a strain was lysogenized with either A3 or A4 the rate at which it absorbed phage PLT 22 (of group A1–A2) was greatly decreased. A derivative lysogenized with an A1–A2 phage absorbed about as fast as before lysogenization; this failure of the A1–A2 phages, which causes a serologically detectable change of the bacterial surface, to diminish phage-absorbing capacity perhaps results from 'form-variation'.

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The Establishment of Lysogeny in *Escherichia coli*. By B. A. Fry and F. Gros (*Institut Pasteur, Paris*)

The adsorption of lambda phage to *Escherichia coli* is known to require the presence of magnesium ions, and we found that if washed cells of *E. coli* K112 are incubated in 0·02 M-$MgSO_4$ with λ_{22} (multiplicity of input 20 particles/cell), it is possible to infect nearly 100 % of the bacteria and when tested for their type of response, 90 % or more of the infected cells are found to be lysogenic. Cells infected in this manner were transferred to a complete amino acid medium containing ^{14}C-phenylalanine and non-utilizable inducer for β-galactosidase, and samples were taken at intervals for the determination of optical density, viable cell count and the synthesis of protein and nucleic acids. The results indicated that in cells giving a lysogenic response, there is a block in the synthesis of protein, ribonucleic acid and deoxyribonucleic acid which lasts for 60–100 min. During this period there is no induced synthesis of β-galactosidase, though the enzyme is readily developed in uninfected cells. Moreover, the synthesis of protein and of nucleic acids all began in the uninfected cells immediately they were placed in the complete medium. Previous workers have shown that in bacteria infected with a temperate phage and giving a lytic response, the synthesis of proteins and nucleic acids still continues. We suggest that the key to the establishment of lysogeny is that in the appropriate conditions, the infecting phage temporarily halts most if not all the synthetic activities of the cell and thus forms an environment in which the genetic material of the phage can attach itself to the bacterial chromosome and thus bring about the establishment of the prophage.

Bacteriophage Lysis on Solid Medium. By Anna Mayr-Harting, (*Department of Bacteriology, Bristol University*)

The diameter of a phage hole grows arithmetically and its growth curve is entirely different from that of the diameter of a bacterial colony. The final size of the hole is a function of the diffusion rate of the phage and its generation time, of the density of the bacterial inoculum and of the thickness of the agar layer. A phage hole may be compared on one hand to a bacterial colony, on the other to a soluble substance diffusing through agar; but it differs from either in the mechanism of its formation.

The number of phage holes developing from aliquots of a phage suspension decreases slowly and steadily with a decrease in the density of the bacterial inoculum.

Phage Multiplication and Toxin Production in *Corynebacterium diphtheriae*. By L. Barksdale (*New York University College of Medicine, New York*)

When diphtherial phage B is grown for one cycle in C4, a non-toxigenic, non-lysogenic, phage-sensitive strain of *Corynebacterium diphtheriae*, toxin and phage are released at the same time. This, then, is toxin production without lysogenicity. Re-examination of lysogenic toxigenic strains such as C 4 (β) and PW 8 (Pdi) reveals that certain agents which induce the change from prophage to phage induce the release of toxin. This suggests that toxin might be a by-product of phage synthesis. Because prophage provides a built-in source of phage, it acts as a gene governing toxigenesis.

The toxigenic diphtheria bacilli which produce the most toxin, while inducible, are defectively lysogenic; those which produce the least are normal lysogenics. The toxigenicity of defective lysogenic strains indicates that phage *maturation* is not necessary for toxigenesis; when phage maturation is inhibited in induced normal lysogenics by the addition of proflavine, the toxin yield is increased.

A low iron level in the medium is necessary for toxin production. Iron deficiency lowers the stability of the lysogenic population 100-fold, thus making induction of prophage to phage frequent; and decreases the burst size tenfold.

When cells are induced to lyse by iron deficiency, toxin and phage both show an initial rise; toxin continues to increase about linearly and without appreciable bacterial growth. The final toxin yield may be 30 times that accompanying the initial burst. If lysis is responsible for toxin release, then the toxin level attained at the end of growth would reflect the accumulation of many random lyses in the iron-deficient population. The fact that diphtherial toxin contains significant amounts of α-ϵ-diaminopimelic acid (a characteristic cell wall component) suggests that toxin may be derived from a part of the bacterium. The enzyme responsible for phage liberation and/or cell lysis may be the toxigenic agent.

Some Studies on the Mechanism of the 'Anaerobic Autolysis' of *Bacillus subtilis*. By W. Kaufmann and K. Bauer (*Biochemical Laboratory, Farbenfabriken Bayer A.G., Wuppertal-Elberfeld, Germany*)

Aerated log-phase cultures of certain strains of *Bacillus subtilis* were shown to autolyse completely within 15–30 min. upon sudden removal of oxygen. Addition of excess Mg^{++} just before removal of O_2 effects the formation of free protoplasts. Crude enzyme solutions prepared from autolysates lysed suspensions of *B. megaterium* and *Sarcina lutea* (Kaufmann & Bauer, 1956).

Moreover, it was recently established that cell walls of *Micrococcus lysodeikticus*, NCTC 2665 (a culture was kindly provided by M. H. Richmond, Cambridge) were lysed by such enzyme solutions. On the basis of activity towards these micro-organisms our enzyme(s) seem(s) to be identical with that found earlier by Richmond (1955, 1957); however, it is probably different from the 'autolysine' of Nomura & Hosoda (1956).

Microscopic studies revealed that as a primary event during the first stage of clarification of cultures of *Bacillus subtilis*, cells 'lysing' most rapidly suffer from plasmoptysis at certain places in the cross-walls resulting in the formation of cylindrical tube-like empty cell walls. On the basis of studied effects of additions of crude 'lytic enzyme', antibiotics, enzyme inhibitors and surface-active agents on well-aerated log-phase cultures and on samples thereof kept in anaerobiosis, one may conclude that: (i) during active cell-respiration the 'lytic enzyme' exists in an inactive, oxidized state and is activated by enzymic reduction upon removal of O_2 or by poisoning the cytochrome-system; (ii) anything that disorganizes or disintegrates the finer structure of the cell membrane provokes the activation of the 'lytic enzyme'; (iii) in the presence of bacteriostatic agents respiration of the cells considerably inhibits the autolysis; (iv) actively respiring and growing organisms must possess some biochemical mechanism, located at the cell surface, protecting the cell wall from the action of an excess of extracellularly added 'lytic enzyme'.

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The Formation of Lysozyme by a Strain of *Bacillus subtilis*. By M. H. RICHMOND (*Department of Biochemistry, University of Cambridge*)

A strain of *Bacillus subtilis*, isolated from soil, releases into the medium during exponential growth an enzyme very similar to egg-white lysozyme (Richmond, 1957). It has been found that the enzyme is very susceptible to surface denaturation at liquid/gas interfaces and consequently becomes inactivated in violently frothing cultures. The enzyme can be stabilized in cultures either by adjusting the rate of shaking to minimize frothing or by the addition of 0·04 % (v/v) *n*-octanol or 0·04 % (v/v) Tween 80. Under these conditions, it can be shown that the level of enzyme in the growth medium at any instant is closely related to the total amount of enzyme in the culture. Consequently, the production of the lytic enzyme in the growth medium provides a measure of synthesis of the enzyme in the whole culture so that this system can be used for studies in protein synthesis.

Creaser (1956) has shown that 8-azaguanine will inhibit the formation of the inducible β-galactosidase in *Staphylococcus aureus*. Similarly, the synthesis of *Bacillus subtilis* lysozyme is completely inhibited by the addition of 50 μg./ml. 8-azaguanine to the synthesizing system. This inhibition can be completely reversed by the further addition of 5 μg./ml. guanine, although the addition of guanine alone to the system has no stimulatory effect.

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Further Studies on the Effect of Linoleic Acid on *Lactobacillus casei*. By E. KODICEK (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

Long-chain unsaturated fatty acids inhibit the growth and metabolic activity of a number of Gram-positive bacteria. This inhibition is reversed by some substances, of which the most potent are 9:10-seco-sterols, such as vitamins D_2 and D_3, and dihydrotachysterol. Vitamin D itself produces an inhibition which in turn can be reversed by linoleic acid. A physico-chemical mechanism has been suggested, namely an alteration of the permeability of the cell membrane and subsequent interference with the active transport of essential metabolites. Another possible explanation was that massive killing was responsible for the inhibitory effect, and that addition of vitamin D removed excess of the bactericidal agent by complexing with it.

To test this possibility, the inhibition and reversal of *Lactobacillus casei* cultures were followed for 24 hr. by viable counts. Addition of linoleic acid, 1·5 μg./ml. medium, produced bacteriostasis, but did not decrease the viable counts which remained constant at the initial level of 10^5. Vitamin D_2, added after 1 hr. incubation with linoleic acid, caused a rapid increase of viable counts, parallel to that of the control cultures. The concentration of linoleic acid was critical, since amounts greater than 2·5 μg./ml. produced an appreciable reduction in viable counts and loss of reversibility. Similarly, the inhibition by vitamin D_2, 8 μg./ml. medium, did not decrease the viable counts for at least 8 hr. Complete reversal occurred on addition of linoleic acid, 2 μg./ml., 1 hr. after incubation with vitamin D_2.

Thus with certain concentrations of linoleic acid and of vitamin D_2, a reversible bacteriostasis can be produced in *Lactobacillus casei*. The effect cannot be due, as the viable counts indicate, to a large-scale killing of bacteria. Hence the concept of a physico-chemical mechanism, affecting the permeability of the cell membrane, still holds good.

Enzymic Lysis of Group A Streptococci. By W. R. MAXTED and H. GOODER (*Central Public Health Laboratory, London*)

The enzyme studied is formed during propagation of a Group C streptococcal bacteriophage (Maxted, 1957) and lyses Group A cells that are resistant to the phage. When produced in a completely dialysable medium it is reasonably stable at room temperature in the pH range 6–8 and is active over this pH range with an optimum value near neutrality. It is precipitated by 40 % saturation with ammonium sulphate and can be dialysed against buffer solutions. A 5- to 10-fold purification has been achieved but with low recovery (5–20 %). It is inactivated at temperatures above 56°. The enzyme needs activation by reducing agents—10^{-3} M-potassium cyanide or sodium thioglycollate being effective.

No proteolytic activity has been found in the enzyme preparations nor in the lysates of the whole cells; nor does streptococcal hyaluronidase affect the lytic reaction.

Enzymic attack on the whole cell or cell walls has been shown serologically to liberate the group hapten and the streptococcal typing antigen (M) in a form not sedimented at 10,000 r.p.m. Rhamnose release from insoluble cell walls has been used as the assay system.

The material released by the enzyme from both group-A cell walls and the variant described by McCarty is a non-dialysable muco complex with the same rhamnose: glucosamine ratio as the original cell-wall substrate. No dialysable constituent has been detected in the material released.

The serologically active carbohydrate precipitated by acetone after removal of the protein by the Sevag method was found to contain the rhamnose, hexosamines, and amino acids usually present in the cell wall.

The enzyme is highly specific for streptococci using wet or acetone-dried preparations and differs from lysozyme, prolysin (endolysin), virolysin and the polysaccharide-depolymerizing enzyme found in Klebsiella phage systems.

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The Valine Carboxyl Group as a Source of Active Formate in *Aerobacter aerogenes*. By M. Webb (*Strangeways Research Laboratory, Cambridge*)

During the period of growth-inhibition, which follows the addition of the folic acid antagonist, Aminopterin, in the early logarithmic phase of a culture of *Aerobacter aerogenes* in a simple chemically defined nutrient solution, alanine, valine and 5-amino-4-imidazolecarboxamide riboside accumulate in the medium. With recovery from the inhibition, which occurs through the inactivation of the antagonist, the accumulated metabolites are utilized rapidly. The amino acids are both used in the synthesis of the carbon skeleton of leucine, the α- and carboxyl carbons of which are derived from the accumulated alanine, and the remaining four carbon atoms from the valine. A study of the utilization of valine in this synthesis has established the elimination of the carboxyl group of this amino acid. Thus, when recovery from Aminopterin-inhibition occurs in the presence of valine-1-^{14}C, none of the carbon isotope is incorporated into leucine. Less than 10 % of the carbon eliminated in this reaction, however, is recovered in the metabolic carbon dioxide; the remainder is incorporated into the nucleic acid purines, and also into thymine, but not into the other pyrimidines, uracil and cytosine. The isotopic carbon incorporated into thymine is located solely in the 5-methyl group, while a significant percentage of the total activity of the guanine is found in C_2 of the purine nucleus. This evidence indicates that the utilization of the valine carboxyl carbon in these synthetic reactions proceeds via 'active' formate. It is suggested that this may be formed together with isobutyrl-CoA by cleavage of α-ketoisovaleric acid, the product of the deamination of valine, in a manner analogous to the production of acetyl-CoA and formic acid from pyruvic acid.

Cobalamin and the Synthesis of Methionine by Cell-free Extracts of *Escherichia coli*.

By C. W. Helleiner, R. L. Kisliuk and D. D. Woods (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

It was shown that treatment by ultrasonic vibration of suspensions of a mutant strain (121–176) of *Escherichia coli* (requiring either methionine or cobalamin for growth) yielded an extract whose synthesis of methionine from homocysteine and serine was increased about fourfold by cyanocobalamin. The addition of a heated extract of *E. coli* was also necessary for optimal synthesis, presumably as a source of a coenzyme form of folic acid required for the transfer of the hydroxymethyl group of serine (Helleiner & Woods, 1956).

It has now been found with similar extracts: (1) that tetrahydropteroylglutamic acid (tetrahydrofolic acid), under anaerobic conditions, is as effective as the heated extract of *Escherichia coli*, (2) that the compound formed by mixing equimolar quantities of formaldehyde and tetrahydropteroylglutamic acid can act directly as the donor of the one-carbon unit to homocysteine (i.e. it replaces both serine and heated extract of *E. coli*), and (3) that, under the conditions of (2), cyanocobalamin is still required for the conversion of homocysteine to methionine. The vitamin (7×10^{-9} M) increased methionine formation 6- to 8-fold in experiments in which the total synthesis varied from 30 to 90 mμmole methionine/mg. protein in 4 hr.; glucose, ATP and DPN were still required for optimal synthesis.

These results are compatible with a view that cobalamin has a function in the transfer of the hydroxymethyl group to homocysteine or in its final reduction to a methyl group.

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The Role of Folic Acid in the Synthesis of Methionine by *Escherichia coli*; the Effect of Cobalamin in the Growth Medium.

By R. L. Kisliuk and D. D. Woods (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

The inclusion of cobalamin in the growth medium of a mutant of *Escherichia coli* (requiring either serine or glycine for growth) endowed extracts of acetone-dried organisms with the ability to utilize tetrahydropteroylglutamic acid (tetrahydrofolic acid) as a carrier of one-carbon units derived from formaldehyde or the β-carbon of serine. Tetrahydropteroylglutamic acid was inactive when no cobalamin was added; the concentration of the latter for a half-maximal effect was 0·7 mμmole/l. growth medium. The action was most pronounced when the organisms were grown in stagnant culture. Addition of cobalamin to the enzymic extract itself had no effect.

Extracts of organisms grown with cobalamin, and depleted of cofactors by dialysis and Dowex-1 treatment, synthesized 60 mμmole L-methionine/mg. protein/hr. when reactivated by tetrahydropteroylglutamic acid. The requirements, in addition to DL-homocysteine, were DPN, ATP, Mg^{++}, hexose diphosphate and inorganic phosphate. Added pyridoxal phosphate was also required absolutely for the utilization of L-serine; no such requirement could be demonstrated when formaldehyde was the substrate. Formaldehyde is therefore not converted to the hydroxymethyl group of serine before reduction to a methyl group.

Under suitable conditions, and with L-serine as donor, tetrahydropteroylglutamic acid promotes the formation of about ten molar equivalents of methionine. Since free formaldehyde reacts non-enzymically with homocysteine to give a metabolically inert substance, it had to be reacted with tetrahydropteroylglutamic acid before addition to the incubation mixture (Kisliuk, 1956).

It appears that a derivative of cobalamin, which is not removed by dialysis or Dowex-1 treatment, is necessary for the conversion of the one-carbon unit of the tetrahydropteroylglutamic acid derivative to the methyl group of methionine.

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A Bacteriostatic Effect in Potato tubers.

By R. Vicente Jordana (*Botany School, University of Cambridge*)

The term 'Cytoarjesis' describes a particular kind of defence mechanism of organisms during processes of cell synthesis. The potato tuber is of special interest for the study of this type of defence, which seems to be of the greatest importance in growing cells and to be fully detectable in embryonic tissues and in those under the influence of meristematic activity. Results from about 4000 observations showed that potato tubers are able to arrest infection when buds are actively developing.

Since the parasite is a living organism capable of infection while it is actively proliferating, it is probable that an interaction of growing activities, ideally reaching an unstable equilibrium, might occur in the host-parasite system. This state of equilibrium is the first condition to be considered for the isolation of the agent.

Antimicrobial activity of normal extracts of potato buds, chicken embryos and some germinated seeds was found. The agent is filterable, does not dialyse, is sensitive to heat and seems to be in a fraction which precipitates with ammonium sulphate. Active suspensions gave absorption at 260–268 mμ. and the presence of DNA and especially of RNA was detected. In optimal conditions of temperature and ionic balance (acid range of pH), inhibition of bacterial growth occurs in a synthetic medium. Sometimes it appears as if self-multiplication was a characteristic of the agent because turbidity has been detected in the absence of bacterial growth. In unsuitable conditions, the agent or a related element behaves as an inductor of bacterial activity. *Bacillus polymyxa* and *Corynebacterium michiganense* proved to be more sensitive to potato extracts.

Possibly the phenomenon would be better understood if a functional biological unit (Cytoarje) were to be established. This should include some ultramicrosomes and viruses, as representatives of normal and abnormal agents of the processes of cell synthesis.

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Fatal Effect of Some Bacterial Toxins on Mice Pre-infected with Mouse Hepatitis Virus (MHV 1). By A. W. Gledhill (*National Institute for Medical Rearch, London*)

It has been shown that previous infection of mice with *Eperythrozoon coccoides*, a harmless blood-parasite of mice, so increases the pathogenic action of MHV 1 that it produces a fatal disease instead of a benign disease. Furthermore, many filtrates of young growths of Gram-negative bacteria have a lethal action when inoculated intraperitoneally into mice which have been suitably pre-infected with *E. coccoides*. The experiments described show that mice also become susceptible to the fatal action of such culture filtrates following infection with MHV 1 and that the increased susceptibility reaches its maximum about one week after inoculation of virus. Mice pre-infected with MHV 1 also resemble mice pre-infected with *E. coccoides* in that they become more susceptible to the toxic effect of lipopolysaccharides extracted from Gram-negative bacteria. The LD_{50} of lipopolysaccharides for pre-treated mice is about 1/10 to 1/100 of that for normal mice and the susceptibility of both normal and pre-infected mice is greater when mice are kept at lower environmental temperatures. The observed relationships between Gram-negative bacterial filtrates, *E. coccoides* and MHV 1, are such that suitably spaced inoculations of any two of them produce a fatal effect upon mice, whereas inoculation of any one of them produces no obvious signs of illness.

DEMONSTRATIONS

Insect Viruses and their Structure. By R. Williams and K. M. Smith (*Agriculture Research Council Virus Research Unit, Cambridge*)

Investigation of the Modes of Action of Antimicrobial Substances; a series of demonstrations by members of the Medical Research Council Unit for Chemical Microbiology and of the Cambridge University Sub-department of Microbial Biochemistry. The demonstrations included: Growth tests and assay of antimicrobial substances (R. Davies); Substances affecting the surface properties of microbial cells (R. Hancock); Micro-electrophoresis studies (R. E. Monro); Inhibition of protein synthesis (M. Richmond and R. Sheinin); Investigation of nucleic acid synthesis (L. V. Crawford); Mutagenic agents (J. C. Shepherd and J. Villanueva); Inhibition of cell-wall synthesis (K. McQuillen); Amino acid incorporation (E. F. Gale); Fractionation of cell components; synchronous growth (B. A. Newton).

The Proceedings included a Symposium on 'Bacterial Lysis' which will be reported elsewhere in this *Journal*.

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